COMBINED EFFECT OF DENSIFICATION AND PRETREATMENT ON CELLULOSIC ETHANOL PRODUCTION

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Combined Effect of Densification and Pretreatment on Cellulosic Ethanol Production

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ABSTRACT

Biomass densification enhances material stability, improves flowability, and decreases both handling and transportation costs. The effect of densification, before or after pretreatment, was tested to determine the effect on cellulosic ethanol processing. Pelleting increased glucose yields of non-pretreated materials by 210% and pelleting followed by acidic and alkaline pretreatments had significant positive impacts on hydrolysis rates or yields. The increase in sugar yields was attributed predominantly to grinding of biomass within the pellet mill. The effects of low pressure densification following AFEX pretreatment were tested under several enzyme loadings both with and without prolonged storage. Densification had no adverse effects on ethanol yields from switchgrass or corn stover; however, prairie cordgrass yields were reduced by 16%. High enzyme loading (15 FPU/g-glucan) produced 15-20% higher ethanol yields than low enzyme loading (5 FPU/g-glucan). Biomass storage by 6-months did not have any negative effects on ethanol yields of AFEX-treated and densified biomass.

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1. BACKGROUND

The rising oil prices, growing concern of environmental pollution, and desire for an alternative outlet for agricultural residues emphasize the need to develop biofuels that are both renewable and environmentally friendly. Transportation is the key oil-consuming sector accounting for 60% of the total oil consumption in 2008 (BPC, 2008). Therefore, significant efforts are made to develop alternative transportation biofuels such as ethanol. Ethanol is a renewable fuel that has potential to replace gasoline. It is currently produced from sugars and starch, and ethanol produced from these routes will continue to be vital. Cellulosic ethanol, however, can be produced using less expensive, renewable, and abundant lignocellulosic biomass as a substrate (Gray et al., 2006). Lignocellulosic biomass includes agricultural and forest residues (Jung et al., 2011), industrial and municipal solid wastes (Jay, 2010), and dedicated energy crops (Digman et al., 2010). Lignocellulosic biomass is investigated as a good source for ethanol production because of larger feedstock availability and its potential to reduce greenhouse gas emissions (Farrell et al., 2006). The Energy Independence and Security Act of 2007 require that 16 billion gallons of cellulosic biofuels must be produced annually by 2022.

However, there are obstacles in economic production of ethanol from lignocellulosic biomass. The logistics of harvesting, handling, transporting, and storing low bulk density lignocellulosic biomass is a primary challenge facing cellulosic ethanol. In addition, bioconversion of lignocellulosic biomass to fermentable sugars and ethanol involves three expensive steps. First, the hemicellulose and lignin barrier in lignocellulosic biomass is broken down or modified to increase the digestibility of cellulose and hemicellulose. Second,

these polysaccharides are broken down to simple sugars. Finally, the fermentable sugars are fermented to produce ethanol.

Several efforts can be made to improve the economic feasibility of cellulosic ethanol production. Densification of lignocellulosic biomass into uniform size prepares biomass for easy handling, enhances the material's stability, improves feedstock flowability, and decreases transportation and storage costs (Hess et al., 2007). Several cost-associated pretreatment technologies have been studied to disrupt the plant cell wall structure to improve bioconversion of lignocelluloses to bioethanol (Agbor et al., 2011; Kim et al., 2011; Tao et al., 2011; Wyman et al., 2011). Hydrolysis with different enzyme types and concentrations has been studied to improve sugar yields (Banerjee et al., 2010; El-Zawawy et al., 2011) and ethanol is produced with higher productivity and concentration using high-yield strains and fermentation techniques such as simultaneous saccharification and fermentation (SSF) (Elkins et al., 2010; Khramtsov et al., 2011).

Recent developments in pretreatment technologies like Ammonia Fiber Expansion (AFEX) coupled with densification processes may enable processing biomass at regional biomass processing centers (RBPCs). RBPC's enables minimizing the distance that low bulk density feedstock bales are transported and enables convenient transportation of densified biomass to a centralized processing plant. In addition, RBPC's reduce the overall biomass handling, transportation, and storage costs. Original biomass (without any pretreatment) can be densified using small pellet mills near the biomass harvesting sites. These densified pellets can be transported to the centralized processing plants to be further processed for ethanol production.

However, most densification processes generate heat that leads to the melting and subsequent solidifying of lignin which acts as a binder in the densification process (Kaliyan and Morey, 2010). During the pelleting process, high temperatures due to the friction between roller and die could potentially degrade the carbohydrates. Any alteration of the lignin structure during the densification process could affect the enzyme's accessibility to the cellulosic fibers. Such impacts can negatively affect the benefits of densification process. Extrusion of soybean hulls negatively affected the sugar release probably because of the interaction of lignin with carbohydrates fibers during extrusion. (Lamsal et al., 2010). Theerarattananoon et. al. (2011) showed that changes in pelleting conditions using different feedstocks improved sugar yields. However, literature on the impact of densification under different densification and pretreatment conditions of biomass feedstock is limited. Thus, it is necessary to evaluate the interaction effect of densification and pretreatment on hydrolysis and fermentation yields of lignocellulosic biomass in addition to evaluating its importance on feedstock logistics.

The overreaching goal of this study is to evaluate interaction effects of biomass pretreatment, densification, and storage on bioconversion efficiency of lignocellulosic biomass for bioethanol production. In this study, the impact of two different densification processes will be investigated. In the first study, switchgrass will be pelleted using a pellet mill and the impact of densification will be documented comparing the hydrolysis yields of original and pelleted biomass after acidic and alkaline pretreatment. In an alternative study, three different biomass samples (switchgrass, corn stover, and prairie cord grass) will be pretreated with the AFEX pretreatment process and densified using ComPAKco densification method (Federal Machine; Fargo, ND). The impact of densification, storage, and enzyme

loadings on AFEX-treated biomass will be studied by testing the SSF yields of stored and non-stored biomass samples in 3 forms (non-pretreated, AFEX-pretreated, and PAKs.

2. LITERATURE REVIEW

2.1. Ethanol

Ethanol is an oxygen-containing organic chemical that is used as a solvent, germicide, beverage, antifreeze, antifreeze, and fuel (Licht, 2006). Currently, ethanol is produced from sugars and starch. Sugar crops such as sugarcane and sugar beet account for about 40% of current global ethanol production and nearly all the remaining ethanol is derived from starch crops. However, cellulosic ethanol is not currently produced on an industrial scale.

The U.S. ethanol market increased from 1.6 billion gallons in 2000 to 12.6 billion gallons in 2010 (RFA, 2010). RFA (2012) reported that increased use of ethanol in US reduced wholesale gasoline prices by an average of $1.09 per gallon and created more than 90,000 direct jobs in 2011. Brazil is the second largest ethanol producing country, and its ethanol market increased to 9 billion gallons in 2008 (Mussatto et al., 2010).

2.2. Lignocellulosic biomass

Lignocellulosic biomass is the non-starchy and fibrous portion of plant materials. Chemical composition of lignocellulosic biomass is a key factor affecting biofuels production and composition varies because of genetic and environmental influences (Hamelinck et al., 2005a). Lignocellulosic biomass primarily consists of cellulose, hemicellulose, and lignin with less than 10% of extractives, protein, and ash (Dehkhoda, 2008). Agricultural residues like grasses contain 25-40% cellulose, 35-50% hemicellulose, and 10-30% lignin (Klass, 1998). Cellulose, hemicellulose, and lignin content of some of the lignocellulosic biomass are reported in the Table 1. Polysaccharides (cellulose and hemicellulose) make up to two-thirds of most lignocellulosic biomass.

Cellulose is a linear and crystalline homopolymer with repeated units of glucose bound together with β-glucosidic bonds. Hemicellulose is a highly branched heteropolymer of xylose, glucose, galactose, mannose, and arabinose. Lignin is a polyphenolic structural constituent of biomass and the largest non-carbohydrate fraction.

The U.S. Department of Energy (2011) affirms that U.S. possesses ample biomass resources (grasses, agricultural wastes, wood wastes, energy crops, etc.) to more than meet our national goals to replace 30% of current petroleum needs (Perlack and Stokes, 2011).

Table 1. Composition of potential lignocellulosic agricultural residues

	cellulose	Hemicellulose	lignin	crude protein	ash	detergent
Crop residues						
Corn Stover	38	26	19	4	5	6
Soybean	33	14	-	14	5	6
Wheat straw	38	29	15	9	4	6
Rye straw	31	25	-	3	3	6
Barley Straw	42	28	-	7	7	11
Warm-season grasses						
Switchgrass	37	29	19	6	3	6
Big bluestem	37	28	18	6	6	6
Indiangrass	39	29	-	6	3	8
Little bluestem	35	31	-	-	-	7
Prairie cordgrass	41	33	-	6	3	6
Miscanthus	43	24	19	-	3	2

Lee, D., Owens, V.N., Boe, A., Jeranyama, P. 2007. Composition of Herbaceous biomass feedstocks. A report submitted to Sun Grant Initiative North Central Center, South Dakota State University

2.3. Preprocessing of biomass

Preprocessing is preparing the biomass for easy handling during transportation, storage, and feeding into processing reactors. It is primarily done by increasing the bulk density, which enhances the material stability, improves feedstock flowability, and decreases the handling and distribution costs (Hess et al., 2009). Biomass may be stored in-field (Huisman

et al., 1997) because of its advantage of low cost. On the other hand, there are significant disadvantages of material loss, self-ignition with increase in moisture level, and microbial degradation (Rentizelas et al., 2009). Some studies have considered intermediate storage locations between the field and the processing plants (Nilsson and Hansson, 2001; Tatsiopoulos and Tolis, 2003). But the use of intermediate storage requires the biomass to be transported twice: first from the farm to the storage location; then, from the storage location to the processing plant. The storage of biomass next to the processing plant has also been examined (Papadopoulos and Katsigiannis, 2002; Tatsiopoulos and Tolis, 2003), but it is also challenged by significant transportation costs. Finally, pretreating and densifying the biomass at intermediate preprocessing locations and transportation of pretreated and densified biomass to the centralized processing plant could be an innovative option.

2.3.1. Densification of biomass

Lignocellulosic biomass holds inherently low bulk densities ranging from 80 to100 kg/m^3 for agricultural residue and grass bales and 150 to 200 kg/m^3 for woody biomass. Thus, these biomass feedstocks are very difficult to handle, transport, and store (Hamelinck et al., 2005b). Densification of biomass into regular shapes could be one way to overcome the above problem. Mechanical compression increases the biomass density by almost ten-fold (Tumuluru et al., 2010). Commercially, biomass densification is performed using pellet mills, extruders, briquetting presses, or roller presses. Pelleting and briquetting are commonly used biomass densification methods. Significant work has been done to study the densification characteristics of agricultural and woody biomass using pellet mills and screw/piston presses (Li and Liu, 2000; Mani et al., 2006b; Ndiema et al., 2002).

Production of quality pellets has been considered more of an art than science (Briggs et al., 1999), and a number of process variables such as die diameter, die temperature, pressure, and use of binders significantly affects the densification process (Granada et al., 2002; Rehkugler and Buchele, 1969). The quality of densified pellets also depends on the chemical composition of the biomass (cellulose, hemicellulos, protein, starch, lignin, crude fiber, fat, and ash) (Granada et al., 2002; Rehkugler and Buchele, 1969).

2.3.1.1. Densification technologies

2.3.1.1.1. Pelletizing

A pellet mill consists of a perforated hard steel die with one or two rollers. There are two types of dies: the ring die and the flat die. Normally, the die remains stationary and the roller rotates, but some rotating die pellet mills are also available (Tumuluru et al., 2010). The biomass feedstock is forced between the roller and die, and rotating die forces the biomass through the perforations to produce densified pellets. Pelleting is a traditional form of densification, and often binders are added to make stable pellets. The process requires high capital investment and high energy inputs. A pellet mill of 400 hp motor could process about 4-5 tons of biomass per hour.

2.3.1.1.2. ComPAKco densification

A novel compaction process called ComPAKco densification developed by Federal Machine in Fargo, ND uses an innovative gear and mesh system to compress biomass through a tapering channel between adjacent gear teeth. This process operates at much lower temperatures compared to high energy wasting and expensive pelleting process. The biomass used for compaction process is pretreated with ammonia fiber expansion (AFEX). AFEX pretreatment is an effective pretreatment technique (Moniruzzaman et al., 1997) that

redistributes the lignin and this lignin acts as a binder even in low temperature generated in the densification process. The capital cost investment for ComPAKco densification is estimated to be less than half of a comparable pelletizer. In addition, this system operates at much lower temperature (60^{o}C) with less biomass degradation compared to pelleting. AFEX pretreated and ComPAKco densified (PAKs) have a bulk density 3-5 times that of baled biomass. This densification process could enable regional biomass processing centers (RBPCs) and reduce the distance that low bulk density biomass will be transported. PAKs potentially eliminate the need of further pretreatment of biomass in a biorefinery. In addition, low moisture content in PAKs enhances the storability and their flowability will enable the use of existing grain handling infrastructure.

2.3.1.2. Densification systems variables

2.3.1.2.1. Process variables

The sturdiness and bulk density of biomass pellets is directly proportional to the operating temperature. The pellet durability of alfalfa increased by 30-35% when the pelleting temperature was increased from 60-104^{o}C (Hill and Pulkinen, 1988). The increase in pressure significantly increases the pellet density (Ndiema et al., 2002). However, pressure above the optimum level may lead to the fractures in briquette because of dilation. In addition, the die geometry has significant impact on the densification process. The energy requirement and the quantity of biomass pelleted are considerably affected with the die size. One study showed that an increase in die thickness increased the glucan content of acid-pretreated biomass (Theerarattananoon et al., 2011).

2.3.1.2.2. Feedstock variables

Water acts as a binding agent by strengthening the van der Waals interaction between the biomass particles (Mani et al., 2006a). However, increasing moisture content above a certain limit (normally 15%) decreases the durability and density of pellets. One study found that corn stover pellets obtained at low moisture content (5-10%) were denser, more stable, and more durable than the pellets obtained at higher moisture content (15%) (Mani et al., 2006a). The optimum initial moisture content for pelleting lignocellulosic biomass is typically 8-12% (Sokhansanj et al., 2005). Lignin, because of its low thermosetting properties and low melting point (140^{o}C), softens and improves the binding ability of lignocellulosic biomass (Kaliyan and Morey, 2010; Van Dam et al., 2004).

2.4. Processing of biomass

2.4.1. Pretreatment

Lignin acts as a structural support for the plants that encloses the carbohydrate polymers. The primary objective of pretreatment is to disrupt this protective lignin and hemicellulose layer of plant cell wall and allow the enzymes access to cellulose for hydrolysis (Sun and Cheng, 2002). Acidic pretreatments lead to removal of hemicellulose, while alkaline pretreatments like ammonia are more effective for lignin solubilization. Physical pretreatments reduce the biomass particle size and cellulose crystallinity in order to increase the specific surface area and reduce the degree of polymerization. These pretreatments affect the overall structure and chemical composition of biomass leading to enhanced enzyme accessibility (McMillan, 1994). However, a good pretreatment technique requires little size reduction, avoids carbohydrate degradation, limits formation of by-products that are inhibitory to hydrolysis and fermentation, and is cost effective (Sun and Cheng, 2002).

Pretreatment is one of the most expensive processing steps for cellulosic ethanol production and costs are estimated at approximately $0.30/ gallon ethanol (Mosier et al., 2005b). Pretreatment technologies that use low-price or recycled catalysts, and/or allow the utilization of lignin co-products is likely to have the lowest net pretreatment costs. Pretreatment methods can be grouped as physical, physico-chemical, acidic methods, and alkaline methods.

2.4.1.1. Physical methods

2.4.1.1.1. Mechanical comminution

Mechanical comminution reduces the particle size and cellulose crystallinity of lignocellulosic biomass in order to increase the specific surface area. This can be done by chipping, grinding, and milling depending on the final particle size (10-30 mm after chipping, 0.2-2 mm after grinding and milling) (Sun and Cheng, 2002). Size reduction is not generally considered a pretreatment method by itself but each pretreatment method requires reduced biomass size to increase effectiveness. Dasari and Berson studied the effect of varying initial particle size on the enzymatic hydrolysis rate of saw dust (Dasari and Berson, 2007). The study showed that particle sizes in the 33-75 µm range compared to 590-850 µm range had 50% higher sugar yields. Yeh et. al. (2010) found that the production of cellobiose was increased 5-fold during enzymatic hydrolysis when microcrystalline cotton cellulose was reduced to submicron scale. Reduction in the particle size will increase the biomass surface to volume ratio (Mansfield et al., 1999) or reduce the cellulose crystallinity (Chang and Holtzapple, 2000) thus improving the enzyme accessibility to cellulosic fibers.

2.4.1.1.2. Extrusion

Extrusion, a well established process in food industries, is studied as a physical pretreatment method for biomass feedstock (Karunanithy and Muthukumarappan, 2010a; Karunanithy et al., 2008; Lamsal et al., 2010). The continuous AFEX treatment and extrusion of corn stover showed significant increase in the sugar yields (Dale et al., 1999). Extrusion of wheat bran had significantly higher sugar yield compared to only particle size reduction (Lamsal et al., 2010). The pretreatment of corn stover and soybean hulls at a solid loading of 75-80% using high shear extrusion process resulted in 54-61% sugar recovery (Karunanithy and Muthukumarappan, 2010b). Increase in barrel temperature and screw speed had a significant positive impact on the sugar release of different biomass feedstocks in an extrusion process (Karunanithy et al., 2008). Although the mechanism of extrusion process in increasing sugar yields is not proven, it can be inferred that the combined effects of reduction in particle size, shear developed during grinding and compression, and thermal softening or plasticization of lignin that occurred during the process of extrusion contributed to improved sugar yields. Reduction in particle size reduces the crystallinity of cellulose (Lamsal et al., 2010) and increases the biomass surface to volume ratio (Mansfield et al., 1999), while shear developed during the extrusion process leads to biomass deconstruction (Karunanithy and Muthukumarappan, 2010a).

2.4.1.2. Physico-chemical methods

2.4.1.2.1. Steam explosion

In steam explosion, biomass is rapidly heated within the temperature range of 160-230^{o}C by high pressure steam and the pressure is reduced suddenly such that the biomass endures an explosive decompression. Hemicellulose is partially hydrolyzed and solubilized, and

lignin is redistributed and removed to some extent (Pan et al., 2005). Hemicellulose removal from the microfibrils is believed to expose the cellulose surface for enzymatic digestibility. This process is not efficient for lignin removal however facilitates melting and redistribution of lignin within the biomass surface (Li et al., 2007).

2.4.1.2.2. Hot water treatment

Hot water treatment is basically cooking of biomass with the temperature in range of 170-230^{o}C and pressure higher than 5 MPa (Sánchez and Cardona, 2008). This pretreatment enlarges the accessible surface area of the biomass by solubilizing the hemicellulose (Zeng et al., 2007). Pretreatment with hot water has the potential to remove 4-22% of cellulose, 35-60% of lignin, and all of the hemicellulose (Kumar et al., 2009; Mosier et al., 2005b). Inoue et. al. (2008) studied the digestibility of eucalyptus with hot-compressed water pretreatment (160^{o}C, 30 min) followed by ball milling (20 min). The study showed approximately 70% yield of total sugar with cellulase loading of 4 FPU/ g substrate. This reported yield was comparable with yield from each of the hot-compressed water and ball milling pretreatments at cellulase loading of 40 FPU/ g substrate. This study showed that combined hot water treatment-ball milling pretreatment can significantly reduce the enzyme loading.

2.4.1.3. Chemical method

2.4.1.3.1. Acid treatment

Acid treatments are more effective for hemicellulose solubilization. Diluted or concentrated acids are used but the concentrated acid is less preferred because of high operational and maintenance costs and production of fermentation inhibitors (Wyman, 1996). Dilute acid pretreatment has been successful at acid concentrations below 4% using a wide variety of lignocellulosic biomass (Kim et al., 2011; Wyman et al., 2011) . Most research is

found on the use of dilute sulfuric acid but nitric acid, phosphoric acid, hydrochloric acid have also been used (Israilides et al., 1978; Mosier et al., 2005a). Wheat straw pretreated with 0.75% v/v of H_2SO_4 at 121^oC for 1 h resulted in a saccharification yield of 74% (Saha et al., 2005). Similar results of 76.5% hydrolysis yield were seen with olive tree biomass at 1.4% H_2SO_4 and 210^oC for 10 min (Cara et al., 2008).

2.4.1.3.2. Alkali treatment

Alkaline pretreatments are more effective for lignin solubilization with minimal losses of cellulose and hemicellulose. These pretreatments are often operated under moderate conditions of temperature and pressure but the pretreatment time is from several hours to days (Kumar and Wyman, 2009b; Mosier et al., 2005b). Hydroxides of sodium, potassium, calcium, and ammonium are the most commonly used reagents for the alkaline pretreatments. This form of pretreatment causes less sugar degradation than acidic pretreatments and is a more effective pretreatment for agricultural residues than hard-wood materials (Kumar and Wyman, 2009b). Alkaline pretreatments disrupt the lignin structure of biomass by swelling. It increases the surface area of cellulose and decreases the degree of crystallinity and polymerization (Taherzadeh and Karimi, 2008).

Soaking in aqueous ammonia (SAA) is an attractive pretreatment technique for agricultural residues (Kim et al., 2008; Ko et al., 2009). Ammonia pretreatment selectively reduces the lignin content of biomass at ambient temperature and pressure. SAA pretreatment of corn stover resulted in 74% delignification and nearly 100% glucan and 85% xylan recovery at room temperature (Kim and Lee, 2005).

2.4.1.3.3. AFEX treatment

Ammonia fiber expansion (AFEX) is the pretreatment process in which biomass is treated with moderate temperatures (60-100^{o}C) and high pressure (10-20 atm) with liquid anhydrous ammonia. The pressure is quickly released causing explosive disruption of biomass. In this method, lignin solubilization and redistribution, and cellulose decrystalization lead to increase in surface area and subsequent hydrolysis yields (Mosier et al., 2005b). AFEX has several advantages which make it of particular interest as a pretreatment. Ammonia used in the process can be recovered and reused (Teymouri et al., 2005), and some remaining serves as a nitrogen supplement in the fermentation process. It is a dry-to-dry process with 100% solid recovery and washing is not required. AFEX treatment does not degrade sugars and almost all cellulose and hemicellulose are preserved (Moniruzzaman et al., 1997). Hydrolysis yields are higher due to increased accessibility of biomass (Galbe and Zacchi, 2007). Despite some phenolic fragments of lignin and cell wall extractives, other inhibitory products are not formed. The major factors affecting the AFEX pretreatment are ammonia loading, temperature, moisture content, and exposure time (Holtzapple et al., 1991). The reported optimal conditions for switchgrass are temperatures of 100^{o}C, ammonia loading rate of one kg of ammonia per kg of dry solid, and a pretreatment time of 5 min. AFEX pretreatment of corn stover had similar optimum operating conditions with the only difference in temperature of 90^{o}C (Kumar et al., 2009).

In contrast to other pretreatment techniques, the lower temperatures and non-acidic conditions of the AFEX process preserves the lignin. During the densification process this lignin serves as a binder.

2.4.2. Hydrolysis

Cellulose molecules are made of long chains of glucose molecules. In hydrolysis, these long chains of polysaccharides are cleaved to release individual sugar monomers, before they are fermented for ethanol production. Cellulose is completely hydrolyzed to glucose while hemicellulose hydrolysis results in the formation of a mixture of pentoses and hexoses. Although acid hydrolysis is possible and required no pretreatment, pretreatment followed by enzymatic hydrolysis is a common approach. Enzymatic hydrolysis has advantages of producing relatively non-toxic hydrolyzates with higher sugar yields.

Enzymatic hydrolysis of cellulose is done with highly specific cellulase and hemicellulase enzymes under mild conditions of temperature (40-50^{o}C) and pH (4-4.5) (Béguin and Aubert, 1994). Cellulases are classified into 3 major classes: endo-glucanases, exo-glucanases, and β-glucosidases. Endoglucanases attack the amorphous region of cellulose and reduces the degree of polymerization to release free chain ends. Exoglucanases shorten the oligosaccharide molecules by binding to the glucan ends and cleaving glucose dimers (cellobiose). Finally the cellobiose is converted to two glucose units by *β*-glucosidases.

The major factors influencing the enzymatic hydrolysis are quality of substrate and its concentration, applied pretreatment method, and the operating conditions like temperature, pH and mixing (Alvira et al., 2010). Hydrolysis reaction rates and yields are higher in low substrate concentration (Cheung and Anderson, 1997). However, the rate of hydrolysis is inhibited at high substrate concentration, and the level of inhibition depends on the ratio of substrate to the total enzyme (Huang and Penner, 1991). Increase in the ratio of enzyme

loading to substrate concentration could enhance the rate of hydrolysis but significantly increases the production costs.

Pretreatment method significantly influence the hydrolysis process (Alvira et al., 2010). Removal of lignin and hemicellulose content has significant impact on the efficiency of enzymatic hydrolysis. Lignin acts as physical barrier of digestible parts of substrate and limits the rate of hydrolysis. In addition, non-productive binding to lignin reduces the activity of cellulolytic enzymes. Hemicellulose removal increases the mean pore size of the substrate and therefore increases the enzyme accessibility.

Enzymatic hydrolysis of biomass could obtain almost 100% cellulose hydrolysis with relatively non-toxic hydrolyzates. However, some disadvantages of enzymatic hydrolysis are longer period of hydrolysis time, high prices of the enzymes, and inhibition of enzymes from released sugars. The cost of enzymes may be reduced by immobilization of cellulase enzymes for recycling.

2.4.3. Fermentation

Fermentation is the process of generating energy from oxidation of organic compounds using an endogenous electron acceptor without the electron transport chain. Ethanol fermentation is a biological process in which sugars (glucose, fructose, sucrose, galactose, and mannose) are converted to pyruvate and conversion of pyruvate to ethanol and CO_2 occurs as metabolic waste products. Pentoses like xylose and arabinose that compose a significant portion of lignocellulosic biomass are not readily fermented by naturally occurring microorganisms, which in turn negatively affect the final ethanol productivity. These C_5 sugars must be used to produce higher ethanol concentrations and reduce the overall cellulosic ethanol costs. Strains of bacteria (Ingram et al., 1998) and yeasts (Ho et

al., 1998) have been genetically modified to co-ferment both hexoses and pentoses to make higher ethanol yields.

2.4.3.1. Separate hydrolysis and fermentation (SHF)

When hydrolysis and fermentation are performed as different steps, it is known as separate hydrolysis and fermentation (SHF). During SHF, hydrolysis is typically performed for the first 48 h at operating temperature for cellulase enzyme (45-50^{o}C) and the temperature is reduced to 30-35^{o}C after 48 h for microorganism (normally yeasts) inoculation to run fermentation. The cellobiose and glucose released during hydrolysis inhibit the cellulase activity reducing rates as hydrolysis proceeds; it is the major drawback of this method. SHF has one advantage of operating the hydrolysis and fermentation at their optimum temperature condition. The operating temperature for hydrolysis is between 45-50^{o}C, depending upon the cellulase-producing microorganisms (Saha et al., 2005), and the operating condition of the fermentation is around 30-37^{o}C, depending on the ethanol producing microorganism.

2.4.3.2. Simultaneous saccharification and fermentation (SSF)

Enzymatic hydrolysis and fermentation can be performed in a single step known as simultaneous saccharification and fermentation (SSF). As stated, glucose and cellobiose produced during biomass hydrolysis inhibits the activity of cellulase. In SSF, sugars in the hydrolyzate are immediately consumed by microorganisms, which prevent cellulase inhibition. Productivity is higher in SSF compared to SHF (Karimi et al., 2006; Nigam and Singh, 1995; Sun and Cheng, 2002). Additionally, the potential of using low enzyme loadings, reduced investment cost due to single reactor processing, avoidance of solid-liquid separation device, and low chance of contamination due to presence of ethanol makes SSF a better fermentation choice. The foremost drawback of this process is indigence to perform

enzymatic hydrolysis and fermentation steps at the same operating conditions of temperature and pH. Wyman (1996) reported that ethanol concentrations of 30 g/l lowered enzyme activity by 25%. SSF can be improved by increasing the substrate loading, lowering the enzyme loadings, and optimizing the temperature and pH.

Economics of ethanol production can be improved by increasing the substrate loading, which increases the ethanol concentration in the reactor. However, high substrate loading tends to decrease the overall ethanol yield. In addition, agitation of high viscosity fermentation broth could be a challenge in high solid SSF process resulting in heat and mass transfer limitations. Fed-batch operation can reduce the extent of inhibition and has advantages of gradual hydrolysis of added fibers (Ballesteros et al., 2002; Rudolf et al., 2005). Enzyme loading impacts the overall economy of cellulosic ethanol. Literature on the precise economics of enzyme loading is limited because of variability in enzyme costs.

SSF is performed at 37 °C, which is the best compromise between the optimum temperature for yeasts (30 °C) and cellulase enzyme (40-45 °C). The compromise in operating temperature is the major drawback of SSF. This limitation can be downsized by the use of thermotolerant yeast strains like *Fabospora fragilis, Saccharomyces uvarum, Candida brassicae, C. lusitaniae, and kluyromyces marxianus,* which allows the temperature closer to the optimum temperature of enzymes (Ballesteros et al., 1991; Ballesteros et al., 2004; Szczodrak and Targoński, 1988).

3. PROBLEM STATEMENT AND OBJECTIVES

3.1. Problem statement

Lignocellulosic biomass possesses inherently low bulk density which cause difficulty in handling, transportation and storage. Densification improves the handling, transportation, storage, flowability, and stability of biomass and reduces associated costs. Bioconversion of lignocellulosic biomass to sugars and ethanol involves 3 processing steps. First, physical and/or chemical pretreatment breaks hemicellulose and/or lignin and crystalline cellulose structure of biomass increasing the enzyme accessibility to cellulosic fibers. Second, cellulose is converted to simple sugars during hydrolysis. Finally, sugars are fermented to ethanol.

Ammonia Fiber Expansion (AFEX) pretreatment technology treats the biomass in moderate temperature (60-100^{o}C) and high pressure (10-20 atm) with liquid anhydrous ammonia. Lignin is one of the primary hindrances to the enzymatic hydrolysis and removal or the transformation of lignin is a main objective of many pretreatment technologies. In the AFEX process, lignin solubilizes and is redistributed on the biomass surface that acts as a binder during ComPAKco densification. Acid pretreatments are more effective for hemicellulose solubilization while, alkaline pretreatments are more effective for lignin solubilization with minimal losses of cellulose and hemicellulose.

Most densification processes generate heat that leads to melting and subsequent solidifying of lignin which allows it to serve as a binder in the densification. The redistribution of lignin during AFEX pretreatment allows the material to be densified under less severe conditions, reducing the energy use and processing costs. Typical biorefinery models include pretreatment of material on site, but AFEX pretreatment may be done at

regional processing centers to reduce overall costs of densification, storage, and transportation. Currently, information on the impact of densification on AFEX pretreated biomass and subsequent hydrolysis and fermentation yields for either fresh or stored biomass is limited.

Biomass densification may also be done prior to pretreatment but the impacts of densification on subsequent pretreatment and hydrolysis are not known. Pelleting is performed prior to biomass pretreatment. High temperatures soften lignin during pelleting so that it acts as a binder in the pelleting process. Any alteration of lignin during densification could potentially impact the efficacy of pretreatment and the enzyme accessibility to cellulosic fibers. Such impacts could counteract the densification benefits of improved feedstock transportation, handling, and storability. The primary objective of this thesis is to determine the combined effect of densification and pretreatment on the enzymatic hydrolysis and fermentation yields.

3.2. Objectives

There will be two major components in my research

1. Evaluate the interaction of biomass pelleting and subsequent pretreatment (using soaking in aqueous ammonia (SAA) and dilute acid pretreatment) and document the impact of densification on overall hydrolysis yield.
2. Evaluate the interaction of biomass pretreatment, densification, and storage on the ethanol yield to determine any advantages or disadvantages of AFEX pretreatment prior to low-pressure densification.

Two projects will be completed to accomplish these research objectives

Objective 1: Combined effect of AFEX pretreatment followed by ComPAKco densification

This is a collaborative project among different universities (South Dakota State University, North Dakota State University, Michigan State University, South Dakota School of Mines and Technology) and industry (Federal Machine, Fargo, ND) that have received funding through the North Central Sun Grant Center. The primary objective of this project is to develop and validate the performance of an integrated biomass pretreatment and densification process that will reduce the logistical hurdles facing second generation biofuels. AFEX pretreatment is being studied with a novel biomass densification process developed by Federal Machine (Fargo, ND). NDSU's research responsibility is to determine the effect of densification and storage on the rates and yields of hydrolysis and fermentation during simultaneous saccharification and fermentation (SSF).

Objective 2: Combined effect of pelleting followed by DA and SAA pretreatment

The primary objective of this study is to determine the impact of biomass pelleting on DA and SAA pretreatments and subsequent hydrolysis.

3.3. Hypotheses

The hypotheses for objective 1

1. Densification of AFEX pretreated biomass does not have adverse effects on the SSF ethanol yields.
2. Storage of ComPAKco-densified biomass (PAKs) does not reduce the SSF ethanol yields.

3. There is a significant difference in SSF ethanol yields between high enzyme loading (15 FPU Spezyme/g-glucan, 64 CBU NS50010/ g-glucan) and low enzyme loading (5 FPU Spezyme/g-glucan, 21.3 CBU NS50010/g-glucan) on PAKs.

The hypotheses for objective 2

4. Pelleting of biomass does not have any negative effect on the DA and SAA pretreatment efficacy.

3.4. References

Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B. 2011. Biomass pretreatment: Fundamentals toward application. Biotechnol. Adv., 29(6), 675-685.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresour. Technol., 101(13), 4851-4861.

Ballesteros, I., Ballesteros, M., CabaÑas, A., Carrasco, J., MartÍn, C., Negro, M., Saez, F., Saez, R. 1991. Selection of thermotolerant yeasts for simultaneous saccharification and fermentation (SSF) of cellulose to ethanol. Appl. Biochem. Biotechnol., 28-29(1), 307-315.

Ballesteros, M., Oliva, J.M., Manzanares, P., Negro, M.J., Ballesteros, I. 2002. Ethanol production from paper material using a simultaneous saccharification and fermentation system in a fed-batch basis. World Journal of Microbiology and Biotechnology, 18(6), 559-561.

Ballesteros, M., Oliva, J.M., Negro, M.J., Manzanares, P., Ballesteros, I. 2004. Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process

(SFS) with Kluyveromyces marxianus CECT 10875. Process Biochemistry, 39(12), 1843-1848.

Banerjee, G., Car, S., Scott-Craig, J.S., Borrusch, M.S., Aslam, N., Walton, J.D. 2010. Synthetic enzyme mixtures for biomass deconstruction: Production and optimization of a core set. Biotechnology and Bioengineering, 106(5), 707-720.

British Petroleum Company (BPC). 2008. Statistical Review of World Energy 2008.

Briggs, J., Maier, D., Watkins, B., Behnke, K. 1999. Effect of ingredients and processing parameters on pellet quality. Poult. Sci., 78(10), 1464-1471.

Béguin, P., Aubert, J.P. 1994. The biological degradation of cellulose. FEMS Microbiology Reviews, 13(1), 25-58.

Cara, C., Ruiz, E., Oliva, J.M., Sáez, F., Castro, E. 2008. Conversion of olive tree biomass into fermentable sugars by dilute acid pretreatment and enzymatic saccharification. Bioresour. Technol., 99(6), 1869-1876.

Chang, V., Holtzapple, M. 2000. Fundamental factors affecting biomass enzymatic reactivity. Appl. Biochem.Biotechnol., 84-86(1), 5-37.

Cheung, S.W., Anderson, B.C. 1997. Laboratory investigation of ethanol production from municipal primary wastewater solids. Bioresour Technol, 59(1), 81-96.

Dale, B., Weaver, J., Byers, F. 1999. Extrusion processing for ammonia fiber explosion (AFEX). Appl. Biochem. Biotechnol., **77**(1), 35-45.

Dasari, R., Berson, E.R. 2007. The effect of particle size on hydrolysis reaction rates and rheological properties in cellulosic slurries. Appl. Biochem. Biotechnol., 137-140(1), 289-299.

Dehkhoda, A. 2008. Concentrating lignocellulosic hydrolysate by evaporation and its fermentation by repeated fedbatch using flocculating Saccharomyces cerevisiae. in: Department of Industrial Biotechnology, MS, Boras University,. Sweden.

Digman, M.F., Shinners, K.J., Casler, M.D., Dien, B.S., Hatfield, R.D., Jung, H.-J.G., Muck, R.E., Weimer, P.J. 2010. Optimizing on-farm pretreatment of perennial grasses for fuel ethanol production. Bioresour. Technol., 101(14), 5305-5314.

El-Zawawy, W.K., Ibrahim, M.M., Abdel-Fattah, Y.R., Soliman, N.A., Mahmoud, M.M. 2011. Acid and enzyme hydrolysis to convert pretreated lignocellulosic materials into glucose for ethanol production. Carbohydrate Polymers, 84(3), 865-871.

Elkins, J.G., Raman, B., Keller, M. 2010. Engineered microbial systems for enhanced conversion of lignocellulosic biomass. Current Opinion in Biotechnology, 21(5), 657-662.

Farrell, A.E., Plevin, R.J., Turner, B.T., Jones, A.D., O'Hare, M., Kammen, D.M. 2006. Ethanol Can Contribute to Energy and Environmental Goals. Science, 311(5760), 506-508.

Galbe, M., Zacchi, G. 2007. Pretreatment of Lignocellulosic Materials for Efficient Bioethanol Production. in: Biofuels, (Ed.) L. Olsson, Vol. 108, Springer Berlin / Heidelberg, pp. 41-65.

Granada, E., López González, L.M., J.L., M., Moran, J. 2002. Fuel lignocellulosic briquettes, die design and products study. Renewable Energy, 27, 561-573.

Gray, K.A., Zhao, L., Emptage, M. 2006. Bioethanol. Current Opinion in Chemical Biology, 10(2), 141-146.

Hamelinck, C.N., Hooijdonk, G.v., Faaij, A.P.C. 2005a. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass and Bioenergy, 28(4), 384-410.

Hamelinck, C.N., Suurs, R.A.A., Faaij, A.P.C. 2005b. International bioenergy transport costs and energy balance. Biomass and Bioenergy, 29(2), 114-134.

Hess, J.R., Wright, C.T., Kenney, K.L. 2007. Cellulosic biomass feedstocks and logistics for ethanol production. Biofuels, Bioproducts and Biorefining, 1(3), 181-190.

Hess, J.R., Wright, C.T., Kenney, K.L., Searcy, E.M. 2009. Uniform-format solid feedstock supply Ssystem: A commodity-scale design to produce an infrastructure-compatible bulk solid from lignocellulosic biomass., Idaho National Laboratory, Idaho Falls, ID, INL/EXT-08-14752.

Hill, B., Pulkinen, D.A. 1988. A study of factors affecting pellet durability and pelleting efficiency in the production of dehydrated alfalfa pellets.

Ho, N.W.Y., Chen, Z., Brainard, A.P. 1998. Genetically Engineered Saccharomyces Yeast Capable of Effective Cofermentation of Glucose and Xylose. Appl. Environ. Microbiol., 64(5), 1852-1859.

Holtzapple, M., Jun, J.-H., Ashok, G., Patibandla, S., Dale, B. 1991. The ammonia freeze explosion (AFEX) process. Appl. Biochem. Biotechnol, 28-29(1), 59-74.

Huang, X., Penner, M.H. 1991. Apparent substrate inhibition of the Trichoderma reesei cellulase system. Journal of Agricultural and Food Chemistry, 39(11), 2096-2100.

Huisman, W., Venturi, P., Molenaar, J. 1997. Costs of supply chains of Miscanthus giganteus. Industrial Crops and Products, 6(3-4), 353-366.

Ingram, L.O., Gomez, P.F., Lai, X., Moniruzzaman, M., Wood, B.E., Yomano, L.P., York, S.W. 1998. Metabolic engineering of bacteria for ethanol production. Biotechnology and bioengineering, 58(2-3), 204-14.

Inoue, H., Yano, S., Endo, T., Sakaki, T., Sawayama, S. 2008. Combining hot-compressed water and ball milling pretreatments to improve the efficiency of the enzymatic hydrolysis of eucalyptus. Biotechnology for Biofuels, 1(1), 2.

Israilides, C.J., Grant, G.A., Han, Y.W. 1978. Sugar level, fermentability, and acceptability of straw treated with different acids. Applied Environmental Microbiology 36 (1), 43–46

Jay, S.G. 2010. National and regional generation of municipal residue biomass and the future potential for waste-to-energy implementation. Biomass and Bioenergy, 34(3), 379-388.

Jung, Y.H., Kim, I.J., Kim, J.J., Oh, K.K., Han, J.I., Choi, I.-G., Kim, K.H. 2011. Ethanol production from oil palm trunks treated with aqueous ammonia and cellulase. Bioresour. Technol., 102(15), 7307-7312.

Kaliyan, N., Morey, R.V. 2010. Natural binders and solid bridge type binding mechanisms in briquettes and pellets made from corn stover and switchgrass. Bioresour. Technol., 101(3), 1082-1090.

Karimi, K., Emtiazi, G., Taherzadeh, M.J. 2006. Ethanol production from dilute-acid pretreated rice straw by simultaneous saccharification and fermentation with Mucor indicus, Rhizopus oryzae, and Saccharomyces cerevisiae. Enzyme and Microbial Technology, 40(1), 138-144.

Karunanithy, C., Muthukumarappan, K. 2010a. Effect of Extruder Parameters and Moisture Content of Switchgrass, Prairie Cord Grass on Sugar Recovery from Enzymatic Hydrolysis. Appl. Biochem.Biotechnol., 162(6), 1785-1803.

Karunanithy, C., Muthukumarappan, K. 2010b. Influence of Extruder Temperature and Screw Speed on Pretreatment of Corn Stover while Varying Enzymes and Their Ratios. Appl. Biochem. Biotechnol., 162(1), 264-279.

Karunanithy, C., Muthukumarappan, K., Julson, J.L. 2008. Influence of high shear bioreactor parameters on carbohydrate release from different biomasses. American Society of Agricultural and Biological Engineers Annual International Meeting 2008. ASABE 084114. ASABE, St. Joseph, Mich.

Khramtsov, N., McDade, L., Amerik, A., Yu, E., Divatia, K., Tikhonov, A., Minto, M., Kabongo-Mubalamate, G., Markovic, Z., Ruiz-Martinez, M., Henck, S. 2011. Industrial yeast strain engineered to ferment ethanol from lignocellulosic biomass. Bioresour. Technol., 102(17), 8310-8313.

Kim, T.H., Lee, Y.Y. 2005. Pretreatment of Corn Stover by Soaking in Aqueous Ammonia. Appl. Biochem. Biotechnol., 1119–1132.

Kim, T.H., Taylor, F., Hicks, K.B. 2008. Bioethanol production from barley hull using SAA (soaking in aqueous ammonia) pretreatment. Bioresour. Technol., 99(13), 5694-5702.

Kim, Y., Mosier, N.S., Ladisch, M.R., Ramesh Pallapolu, V., Lee, Y.Y., Garlock, R., Balan, V., Dale, B.E., Donohoe, B.S., Vinzant, T.B., Elander, R.T., Falls, M., Sierra, R., Holtzapple, M.T., Shi, J., Ebrik, M.A., Redmond, T., Yang, B., Wyman, C.E., Warner, R.E. Comparative study on enzymatic digestibility of switchgrass varieties

and harvests processed by leading pretreatment technologies. Bioresour. Technol. 102:11089–11096.

Klass, D.L. 1998. Biomass for renewable energy, fuels, and chemicals., San Diego, Academic Press.

Ko, J.K., Bak, J.S., Jung, M.W., Lee, H.J., Choi, I.G., Kim, T.H., Kim, K.H. 2009. Ethanol production from rice straw using optimized aqueous-ammonia soaking pretreatment and simultaneous saccharification and fermentation processes. Bioresour. Technol., 100(19), 4374-4380.

Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P. 2009. Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. Industrial & Engineering Chemistry Research, 48(8), 3713-3729.

Kumar, R., Wyman, C.E. 2009. Effects of cellulase and xylanase enzymes on the deconstruction of solids from pretreatment of poplar by leading technologies. Biotechnology Progress, 25(2), 302-314.

Lamsal, B., Yoo, J., Brijwani, K., Alavi, S. 2010. Extrusion as a thermo-mechanical pre-treatment for lignocellulosic ethanol. Biomass and Bioenergy, 34(12), 1703-1710.

Li, J., Henriksson, G., Gellerstedt, G. 2007. Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion. Bioresour. Technol., 98(16), 3061-3068.

Li, Y., Liu, H. 2000. High-pressure densification of wood residues to form an upgraded fuel. Biomass and Bioenergy, 19(3), 177-186.

Licht, F.O. 2006. World Ethanol Market: The Outlook to 2015, Agra Europe Special Report, UK.

Mani, S., Tabil, L.G., Sokhansanj, S. 2006a. Effects of compressive force, particle size and moisture content on mechanical properties of biomass pellets from grasses. Biomass and Bioenergy, 30(7), 648-654.

Mani, S., Tabil, L.G., Sokhansanj, S. 2006b. Specific energy requirement for compacting corn stover. Bioresour. Technol., 97(12), 1420-1426.

Mansfield, S.D., Mooney, C., Saddler, J.N. 1999. Substrate and Enzyme Characteristics that Limit Cellulose Hydrolysis. Biotechnology Progress, 15(5), 804-816.

McMillan James, D. 1994. Pretreatment of Lignocellulosic Biomass. in: Enzymatic Conversion of Biomass for Fuels Production, Vol. 566, American Chemical Society, 292-324.

Moniruzzaman, M., Dale, B., Hespell, R., Bothast, R. 1997. Enzymatic hydrolysis of high-moisture corn fiber pretreated by AFEX and recovery and recycling of the enzyme complex. Appl. Biochem.Biotechnol., 67(1), 113-126.

Mosier, N., Hendrickson, R., Ho, N., Sedlak, M., Ladisch, M.R. 2005a. Optimization of pH controlled liquid hot water pretreatment of corn stover. Bioresour. Technol., 96(18), 1986-1993.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M. 2005b. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol., 96(6), 673-686.

Mussatto, S.I., Dragone, G., Guimarães, P.M.R., Silva, J.P.A., Carneiro, L.M., Roberto, I.C., Vicente, A., Domingues, L., Teixeira, J.A. 2010. Technological trends, global market, and challenges of bio-ethanol production. Biotechnology Advances, 28(6), 817-830.

Ndiema, C.K.W., Manga, P.N., Ruttoh, C.R. 2002. Influence of die pressure on relaxation characteristics of briquetted biomass. Energy Conversion and Management, 43(16), 2157-2161.

Nigam, P., Singh, D. 1995. Enzyme and microbial systems involved in starch processing. Enzyme and Microbial Technology, 17(9), 770-778.

Nilsson, D., Hansson, P.A. 2001. Influence of various machinery combinations, fuel proportions and storage capacities on costs for co-handling of straw and reed canary grass to district heating plants. Biomass and Bioenergy, 20(4), 247-260.

Pan, X., Xie, D., Gilkes, N., Gregg, D., Saddler, J. 2005. Strategies to enhance the enzymatic hydrolysis of pretreated softwood with high residual lignin content. Appl. Biochem.Biotechnol, 124(1), 1069-1079.

Papadopoulos, D.P., Katsigiannis, P.A. 2002. Biomass energy surveying and techno-economic assessment of suitable CHP system installations. Biomass and Bioenergy, 22(2), 105-124.

Perlack, R.D., Stokes, B.J. 2011. U.S. Billion-Ton Update: Biomass Supply for a Bioenergy and Bioproducts Industry. Oak Ridge National Laboratory, Oak Ridge, TN.

Rehkugler, G.E., Buchele, W.F. 1969. Biomechanics of forage wafering. Transactions of the ASAE, 12, 1-8.

Rentizelas, A.A., Tolis, A.J., Tatsiopoulos, I.P. 2009. Logistics issues of biomass: The storage problem and the multi-biomass supply chain. Renewable and Sustainable Energy Reviews, 13(4), 887-894.

RFA. 2010 Ethanol Industry outlook:climate and opportunity.

Rudolf, A., Alkasrawi, M., Zacchi, G., Lidén, G. 2005. A comparison between batch and fed-batch simultaneous saccharification and fermentation of steam pretreated spruce. Enzyme and Microbial Technology, 37(2), 195-204.

Saha, B.C., Iten, L.B., Cotta, M.A., Wu, Y.V. 2005. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochemistry, 40(12), 3693-3700.

Sokhansanj, S., Mani, S., Bi, X., Zaini, P., L., T. 2005. Binderless Pelletization of Biomass. ASAE Annual International Meeting, Tampa, FL.

Sun, Y., Cheng, J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour. Technol., 83(1), 1-11.

Szczodrak, J., Targoński, Z. 1988. Selection of thermotolerant yeast strains for simultaneous saccharification and fermentation of cellulose. Biotechnology and Bioengineering, 31(4), 300-303.

Sánchez, Ó.J., Cardona, C.A. 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour. Technol., 99(13), 5270-5295.

Taherzadeh, M., Karimi, K. 2008. Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production: A Review. International Journal of Molecular Sciences, 9(9), 1621-1651.

Tao, L., Aden, A., Elander, R.T., Pallapolu, V.R., Lee, Y.Y., Garlock, R.J., Balan, V., Dale, B.E., Kim, Y., Mosier, N.S., Ladisch, M.R., Falls, M., Holtzapple, M.T., Sierra, R., Shi, J., Ebrik, M.A., Redmond, T., Yang, B., Wyman, C.E., Hames, B., Thomas, S., Warner, R.E. 2011. Process and technoeconomic analysis of leading pretreatment

technologies for lignocellulosic ethanol production using switchgrass. Bioresour. Technol., 102(24), 11105-11114.

Tatsiopoulos, I.P., Tolis, A.J. 2003. Economic aspects of the cotton-stalk biomass logistics and comparison of supply chain methods. Biomass and Bioenergy, 24(3), 199-214.

Teymouri, F., Laureano-Perez, L., Alizadeh, H., Dale, B.E. 2005. Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover. Bioresour. Technol., 96(18), 2014-8.

Theerarattananoon, K., Xu, F., Wilson, J., Staggenborg, S., McKinney, L., Vadlani, P., Pei, Z., Wang, D. Effects of the pelleting conditions on chemical composition and sugar yield of corn stover, big bluestem, wheat straw, and sorghum stalk pellets. Bioprocess and Biosystems Engineering, 35(4), 1-9.

Tumuluru, J.S., Wright, C.T., L., K.K., J., H.R. 2010. A Review on Biomass Densification Technologies for Energy Application. Biofuels, Bioprod. Biorefin., 2011, 5, 683–707.

Van Dam, J.E.G., Van den Oever, M.J.A., Teunissen, W., Keijsers, E.R.P., Peralta, A.G. 2004. Process for production of high density/high performance binderless boards from whole coconut husk: Part 1: Lignin as intrinsic thermosetting binder resin. Industrial Crops and Products, 19(3), 207-216.

Weil, J., Sarikaya, A., Rau, S.L., Goetz, J., Ladisch, C., Brewer, M., Hendrickson, R., Ladisch, M. 1997. Pretreatment of yellow poplar sawdust by pressure cooking in water. Appl. Biochem.Biotechnol, 68(1), 21-40.

Wyman, C.E. 1996. Handbook on Bioethanol: Production and Utilization. Taylor Francis, Washington.

Wyman, C.E., Balan, V., Dale, B.E., Elander, R.T., Falls, M., Hames, B., Holtzapple, M.T., Ladisch, M.R., Lee, Y.Y., Mosier, N., Pallapolu, V.R., Shi, J., Thomas, S.R., Warner, R.E. 2011. Comparative data on effects of leading pretreatments and enzyme loadings and formulations on sugar yields from different switchgrass sources. Bioresour. Technol., 102(24), 11052-11062.

Xu, J., Cheng, J.J. 2011. Pretreatment of switchgrass for sugar production with the combination of sodium hydroxide and lime. Bioresour. Technol., 102(4), 3861-3868.

Yeh, A.I., Huang, Y.-C., Chen, S.H. 2010. Effect of particle size on the rate of enzymatic hydrolysis of cellulose. Carbohydrate Polymers, 79(1), 192-199.

Zeng, M., Mosier, N.S., Huang, C.P., Sherman, D.M., Ladisch, M.R. 2007. Microscopic examination of changes of plant cell structure in corn stover due to hot water pretreatment and enzymatic hydrolysis. Biotechnology and Bioengineering, 97(2), 265-278.

4. PAPER 1. COMBINED EFFECT OF PELLETING AND PRETREATMENT ON ENZYMATIC HYDROLYSIS OF SWITCHGRASS[1, 2]

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4.1. Abstract

Switchgrass was pelleted to evaluate the effect of densification on acidic and alkaline pretreatment efficacy. Bulk density and durability of pellets were 724 kg/m^3 and 95%, respectively. Ground switchgrass (D_{90} = 21.7 mm) was further ground to a fine power (D_{90} = 0.5 mm) in the pellet mill prior to densification. This grinding increased enzymatic hydrolyzate glucose yields of non-pretreated materials by 210%. Pelleting had no adverse impact on dilute acid pretreatment efficacy. Grinding and pelleting increased hydrolyzate glucose yields of switchgrass pretreated by soaking in aqueous ammonia (SAA) by 37%. Xylose yields from SAA-pretreated switchgrass pellets were 42% higher than those from the original biomass. Increases in sugar yields from SAA-pretreated pelleted biomass are attributed to grinding and heating of biomass during the pelleting process. Potential transportation, storage, and handling benefits of biomass pelleting may be achieved without negatively affecting the downstream processing steps of pretreatment or enzymatic hydrolysis.

4.2. Keywords

Densification, Particle size, Dilute acid, Aqueous ammonia, Lignin

4.3. Introduction

Low densities of biomass feedstocks and the associated handling, transportation, and storage costs are major impediments to the utilization of biomass for biofuel production. The densification of biomass into uniform pellets could be one method to reduce these challenges. Pelleting increases the biomass density by almost ten-fold (Tumuluru et al., 2011) thereby facilitating easy handling and decreasing transportation and storage costs (Hess et al., 2007).

Significant work has been done to study the densification characteristics of agricultural and woody biomass (Kaliyan and Morey, 2010; Larsson et al., 2008; Mani et al., 2006).

The recalcitrant structure of lignocellulosic biomass is another challenge to commercial bioethanol production. Expensive pretreatment is needed for disruption of cell wall structures to make polysaccharides more accessible to enzymes for effective hydrolysis. Several cost-effective methods including dilute acid (DA) and soaking in aqueous ammonia (SAA) pretreatments have been developed to disrupt plant cell wall structures for efficient bioconversion of lignocellulosic biomass to fermentable carbohydrates (Tao et al., 2011; Wyman et al., 2011).

Chemical pretreatment of biomass increases the biomass surface area and pore volume through delignification, hemicellulose solubilization, and reduction in cellulose crystallinity (McMillan, 1994). Acid treatments are more effective for hemicellulose solubilization while alkaline pretreatments are more effective for lignin solubilization with minimal losses of cellulose and hemicellulose. Physical pretreatments of biomass reduce particle size and cellulose crystallinity in order to increase the specific surface area and reduce the degree of polymerization. Extrusion has been shown to be an effective pretreatment for many forms of biomass (Karunanithy and Muthukumarappan, 2010; Karunanithy et al., 2008), although in one case it negatively affected sugar release from soybean hulls (Lamsal et al., 2010). This effect was attributed to the interaction of lignin with carbohydrate fibers during extrusion.

Pelleting increases biomass bulk density through mechanical and thermal processing similar to extrusion (Larsson et al., 2008). High temperatures generated in the pelleting process soften lignin and enable it to act as a binder to form durable pellets (Kaliyan and Morey, 2010). Because lignin content and distribution has a strong influence on biomass

recalcitrance, alteration of lignin during densification could impact pretreatment efficacy or hydrolysis yields. Theerarattananoon et al. (2011) showed that changes in pelleting parameters can influence sugar yields.

Since literature on the impact of pelleting under different pelleting and pretreatment conditions of biomass feedstock is limited, the present study used switchgrass as a model substrate to investigate the interaction of densification and pretreatment. Switchgrass is a promising feedstock for bioethanol production (Hu et al., 2010; Isci et al., 2008) because of its high productivity, suitability for growth on marginal land, and environmental benefits (McLaughlin and Taliaferro, 1999). DA and SAA pretreatments are effective methods to increase hydrolysis yields from switchgrass (Isci et al., 2008; Tao et al., 2011; Wyman et al., 2011). They were used as model pretreatments to demonstrate interaction between densification for acidic and alkaline pretreatments. The overall objective of this study was to evaluate the impact of pelleting on the efficacy of acidic and alkaline pretreatments for enhancing enzymatic hydrolysis of switchgrass.

4.4. Materials and Methods

4.4.1. Materials

Sunburst switchgrass (*Panicum virgatum L.*) was harvested in the fall of 2008 and obtained from North Dakota State University's (NDSU) Central Grassland Research and Extension Center in Streeter, ND. The switchgrass was ground in a model 915 hammermill (CrustBuster/ Speedking, Inc.; Dodge City, KS, USA) fitted with an 8-mm sieve. This original ground material at 5% moisture content (dry basis (db)) was stored in a sealed plastic bag at room temperature. Reagent grade glucose and xylose were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ, USA). The enzymes, NS50013 (endo/exo-

cellulase, activity: 77.0 filter paper units (FPU)/mL), Novozyme 188 (β-glucosidase, activity: 500.5 cellobiase units (CBU)/mL), and Cellic HTec (endo-xylanase, 10,596 IU/mL) were provided by Novozymes North America, Inc. (Franklinton, NC, USA). The cellulose and cellobiase activities were determined using the method described by Ghose (1984) while xylanase activity of Cellic HTec was determined as described by Bailey et al. (1992).

4.4.2. Pellet production

Ground switchgrass was pelleted using a Buskirk Engineering pellet mill (PM 810; North Ossian, IN) in the NDSU Biomass Feedstock Processing Laboratory at the USDA-ARS Northern Great Plains Research Laboratory (Mandan, ND). The pellet mill had a 7.5-kW motor and a capacity to produce 180 kg of biomass pellets per hour. The 200-mm (diameter) ×38-mm (thickness) plate die had holes 6.3 mm in diameter. The moisture content of the original biomass was adjusted to 12% db prior to feeding into the pellet mill. Additionally, biomass in the hopper was wetted lightly using a spray bottle to aid pelleting. The original switchgrass was ground to a fine powder within the pellet mill prior to entering the die to produce pellets. Samples of this powdered material were used to distinguish impacts of fine grinding and subsequent pelleting on pretreatment efficacy. No external binders were added while pelleting. The pellets (4 kg) were collected and stored in plastic bags at room temperature. Glucose and xylose yields of the original, powdered, and pelleted biomass were compared for three pretreatments (non-pretreated, DA-pretreated, and SAA-pretreated) to evaluate the interaction of pelleting and pretreatments.

4.4.3. Bulk density and durability of pellets

Bulk density of pellets was determined using ASABE Standard S269.4 (ASABE, 2007). Durability is defined as the ability of densified biomass to remain intact when handled and

was determined using ASABE Standard S269.4 (ASABE, 2007). Pellet samples were prepared by sieving through a 4-mm aperture sieve to remove fines. Triplicate 500-g samples of sieved pellets were used for testing. After 10 min of tumbling at 50 rpm, the samples were removed and sieved again using a 4-mm sieve. Durability was calculated as the ratio of total sieved pellet mass after tumbling to mass prior to tumbling.

4.4.4. Determination of particle size and size distribution of original and powdered biomass

Original switchgrass (milled through 8-mm mesh screen) and powdered biomass (ground in pellet mill between the rollers and die prior to compression and pellet formation) were used as control materials. The machine vision approach as reported by Igathinathane et al. (2012) was used to determine the size distribution of particulate material. A flatbed scanner was used to obtain digital images of milled and powdered biomass. A user-coded ImageJ plugin was used for analyzing the digital images for the size and size distribution of these particulate materials. The particle size distribution (PSD) was analyzed based on the length and ∑Volume approach (Igathinathane et al., 2012).

4.4.5. DA pretreatment

DA pretreatment was conducted in a 1-L Parr pressure reactor (4600 Series, Parr Instrument Company; Moline, IL, USA). Biomass samples in original, powdered, and pelleted forms (50 g (db)) were mixed with dilute sulfuric acid (1.5% (w/w)) at a solid loading rate of 10% (w/v). The samples were treated at 140 °C for 20 min. The time to reach the desired temperature (140 °C) was approximately 60 min for all samples and the resulting pressure was 275 kPa. After holding for 20 min at the set temperature, the reaction vessel was immediately transferred to an ice-water bath until the inside pressure was equalized to

that of the atmosphere. Pellets were completely disintegrated during pretreatment. The DA-pretreated biomass was washed with distilled water (3 L) in a vacuum-assisted Buchner funnel through Whatman #41 filter paper (20-25 μm pore size). Samples of supernatant were collected to analyze individual sugars via high performance liquid chromatography (HPLC). The solids remaining after filtration were weighed and stored in a sealed plastic bag at 4 °C. A fraction of pretreated and washed biomass was used for determining moisture content and chemical composition.

4.4.6. SAA pretreatment

Switchgrass samples in each of the three biomass forms were pretreated by SAA using 15% ammonium hydroxide (EMD Chemicals Inc., Gibbstown, NJ, USA). The pretreatment was performed in screw-capped Pyrex bottles (1-L) at a solid-to-liquid ratio of 1:6 at 40 °C for 24 h without agitation. Biomass pellets were completely disintegrated during pretreatment. The pretreated biomass was washed with distilled water (3 L) in a vacuum-assisted Buchner funnel through Whatman #41 filter paper (20-25 μm pore size). The washed solids were collected and stored at 4 °C. The solid recovery of pretreated biomass was calculated by drying a small fraction of wet solids overnight in a convection oven at 105 °C.

4.4.7. Composition analysis

Compositional analysis was performed for non-pretreated, DA-pretreated, and SAA-pretreated switchgrass in the original, powdered, and pelleted forms. Carbohydrate and lignin (acid-soluble and acid-insoluble) contents were calculated using National Renewable Energy Laboratory (NREL) Chemical Analysis and Testing Standard Procedures (Sluiter et al., 2008b). The extractives were removed from the non-pretreated biomass following the NREL

Chemical Analysis and Testing Procedures (Sluiter et al., 2008a). Each analysis was performed in triplicate for all samples.

4.4.8. Enzymatic hydrolysis

Enzymatic hydrolysis of biomass samples was performed in 250-mL Erlenmeyer flasks with 100 mL of working volume. An initial glucan loading of 1% was used for hydrolysis. The biomass samples were mixed with sodium citrate buffer (50 mM, pH 4.8) and agitated in a water bath shaker (MaxQ 7000, Thermo Scientific; Dubuque, IA, USA) at 50 °C and 130 rpm for 72 h. Each flask was supplemented with sodium azide (0.04% (w/v)) to prevent microbial contamination during hydrolysis. The enzymes, cellulase, cellobiase, and xylanase were added at loading rates of 25 FPU/g glucan, 50 CBU/g glucan and 3500 IU/ g glucan, respectively. Samples (1 mL) were taken every 24 h and centrifuged at 13800 x g for 5 min (Galaxy 16 micro-centrifuge, VWR International; Bristol, CT, USA). After centrifugation, the supernatant was filtered through a 0.2-µm nylon filter (Pall Corporation; West Chester, PA) and stored at -20 °C until analysis. All hydrolysis experiments were done in triplicate.

4.4.9. HPLC

Hydrolysis samples were analyzed via an HPLC instrument (Waters Corp.; Milford, MA, USA) equipped with an autosampler, an isocratic pump, and a refractive index detector (model 2414; Waters Corp.). The hydrolyzed samples were analyzed for sugars using an Aminex HPX-87P (300×7.8 mm) carbohydrate column (Bio-Rad Laboratories; Hercules, CA) with a mobile phase of 18 mΩ NANO pure water at a flow rate of 0.6 mL/min at 85°C.

4.4.10. Statistical analysis

The General Linear Model, PROC GLM procedure in SAS (version 9.2, SAS Institute, Inc., Cary, N.C.) was used to determine least significant difference (LSD) values at $p<0.05$.

The tests of significant difference of overall means were performed for chemical composition (glucan, xylan, and lignin) and glucose yields (at 24 h and 72 h) of the original, powdered, and pelleted biomass samples among the group of non-pretreated, DA-pretreated, and SAA-pretreated biomass.

4.5. Results and Discussion

4.5.1. Size distribution and composition of switchgrass samples

The PSD parameters of original and powdered switchgrass are shown in Table 1. Particle size was reduced by more than 98% and uniformity index and coefficient of uniformity reveal that the powdered biomass sample contained slightly more uniform particles than the original sample.

The chemical composition of non-pretreated, DA-pretreated, and SAA-pretreated switchgrass samples in original, powdered, and pellet forms is shown in Table 2. The glucan, xylan, and lignin contents of non-pretreated original switchgrass were comparable with previously reported values (Hu et al., 2010; Yan et al., 2010). Neither grinding nor pelleting had a significant impact on the chemical composition of the non-pretreated switchgrass. Similar findings have been reported with wheat straw, corn stover, big bluestem, and sorghum stalks (Theerarattananoon et al., 2011).

Total solid recoveries of 46% to 59% were achieved during DA-pretreatment of switchgrass samples. Solids loss during DA-pretreatment was mainly attributed to xylan removal. Xylan removal was greater than 93% for all the biomass forms. This loss in xylan is typical during DA pretreatment (Cara et al., 2008), and it led to increases in glucan and lignin contents. There was no significant difference in the glucan, xylan, and lignin contents of the different forms of DA-pretreated biomass.

Table 2. Particle size distribution (PSD) parameters of ground switchgrass

PSD parameters	Original biomass*	Powder biomass*
D_{90}	21.71	0.50
D_{84}	18.96	0.28
D_{75}	11.98	0.18
D_{50}	7.69	0.10
D_{25}	4.79	0.07
D_{10}	2.62	0.05
Geometric mean length (GML)**	7.16	0.12
GML Standard Deviation**	2.34	2.47
Uniformity index (%) ***	7.05	8.36
Coefficient of uniformity§ (decimal)	3.61	2.55
Size range variation coefficient (%)§§	100.37	109.58

*All dimensions in mm, unless specified otherwise ; ** Calculated based on ASABE Standards 424.1(ASABE, 2007b) following the machine vision method (Igathinathane et al., 2012); *** D_5/D_{90} – increased value indicates uniform particles (Igathinathane et al., 2009); § D_{60}/D_{10} – reduced value indicates uniform particles (Igathinathane et al., 2009); §§ $((D_{84} - D_{16})/2D_{50}) \times 100$ – increased value indicates wider range of dimensional variation and less uniform particles (Igathinathane et al., 2009)

Table 3. Chemical composition of switchgrass as affected by pelleting, SAA, and DA pretreatments

	Component in solid fractions (%)		
Biomass Feedstock	Glucan (%)	Xylan (%)	Lignin (%)
Untreated			
Original	36[a]	20.7[a]	23.2[a]
Powder	38.8[a]	21.4[a]	21.6[a]
Pellet	35.1[a]	19.4[a]	22.7[a]
DA-Pretreated			
Original	64.8[a]	2.4[a]	35[a]
Powder	62.5[a]	2.2[a]	33.3[a]
Pellet	60.8[a]	2.6[a]	35.1[a]
SAA-Pretreated			
Original	41.7[a]	23.9[a]	18.9[a]
Powder	46[b]	22.9[a]	19.8[a]
Pellet	41.1[a]	19[b]	19.9[a]

Significant differences ($p<0.05$) for each component within a feedstock category are denoted by different letters

During SAA pretreatment, 75% to 85% of the original solid was retained. Solids loss during this pretreatment was mainly attributed to lignin and xylan removal. Except for the glucan content of powdered biomass and the xylan content of pelleted biomass, other chemical constituents in the group of original, powdered, and pelleted biomass samples were statistically similar ($p<0.05$). Overall, the grinding and heating involved in the intermediate powdering and subsequent pelleting did not significantly impact the chemical composition of switchgrass samples or their composition following pretreatment.

4.5.2. Effect of pelleting on enzymatic hydrolysis of non-pretreated switchgrass

The glucose concentrations during enzymatic hydrolysis of non-pretreated original, powdered, and pellet forms of biomass are shown in Fig.1. The powdered biomass had glucose yields significantly greater than those from the original biomass indicating that grinding within the pellet mill acts as a form of pretreatment. Pelleted biomass had a slightly lower glucose yield (~5%) than the powdered form but yields were still significantly higher than those of the original biomass. The pelleted biomass was not disintegrated completely at 72 h and likely contributed to lower glucose yields compared to that from powdered biomass. Increases in glucose yields from finely ground biomass have been attributed to thermal degradation and reduction in crystallinity during mechanical processing (Karunanithy and Muthukumarappan, 2010; Karunanithy et al., 2008; Lamsal et al., 2010; Millett et al., 1979). Dasari and Berson (2007) found a 50% increase in glucose yields for particle sizes in the 33-75 µm range compared to particles of 590-850 µm. This indicates that sugar yields from powdered biomass (D_{90} = 0.5 mm) used in this study could further improve with additional grinding. Given that thermochemical pretreatment of lignocellulosic biomass is an expensive

process, pelleting lignocellulosic biomass may be used as a sole pretreatment technique or as a technique to reduce severity of further thermochemical pretreatments.

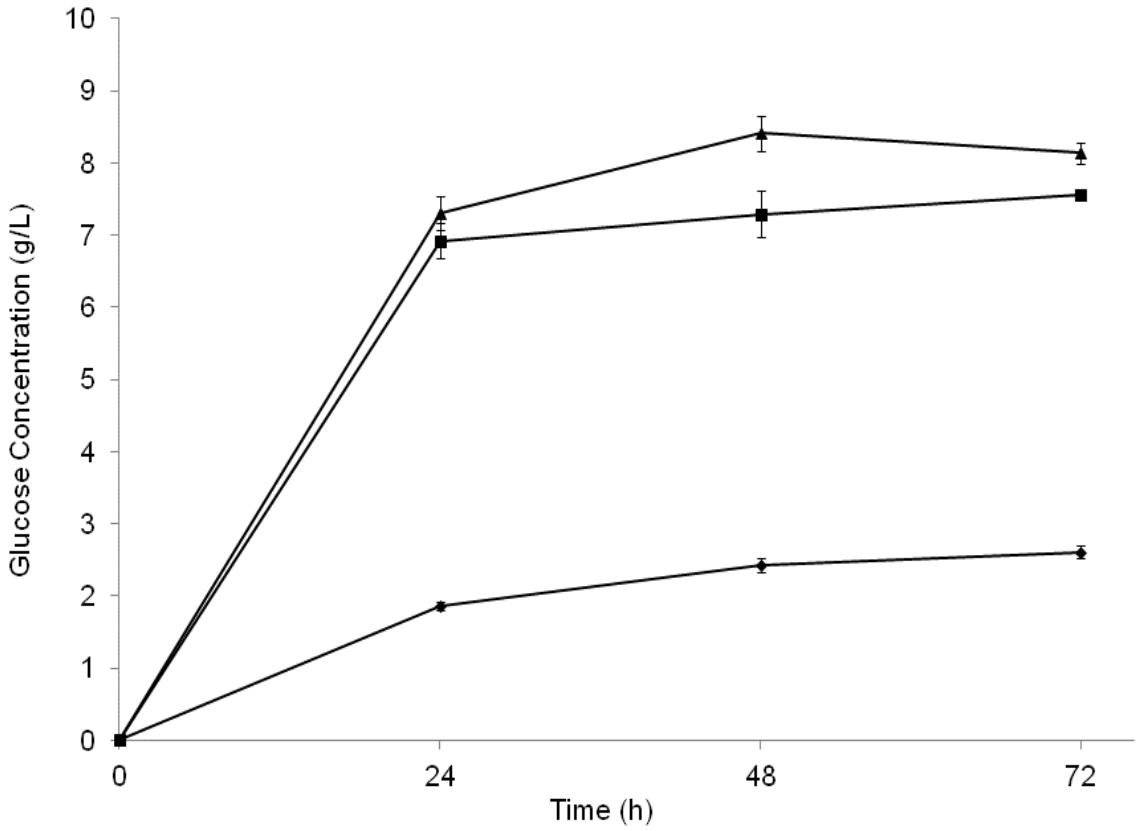

Figure 1: Effect of pelleting on glucose yields from enzymatic hydrolysis of non-pretreated switchgrass.
Data points are average values for triplicate reactors and error bars represent sample standard deviation. ♦: Original; ■: Powder; ▲: Pellet

The xylose yields from non-pretreated original, powdered, and pelleted forms of biomass are shown in the Fig. 2. Trends of xylose production were similar to those of glucose yields. Xylose yields of powdered biomass increased by 29% compared to original biomass and pelleted biomass had an 8% lower xylose yield compared to powdered biomass.

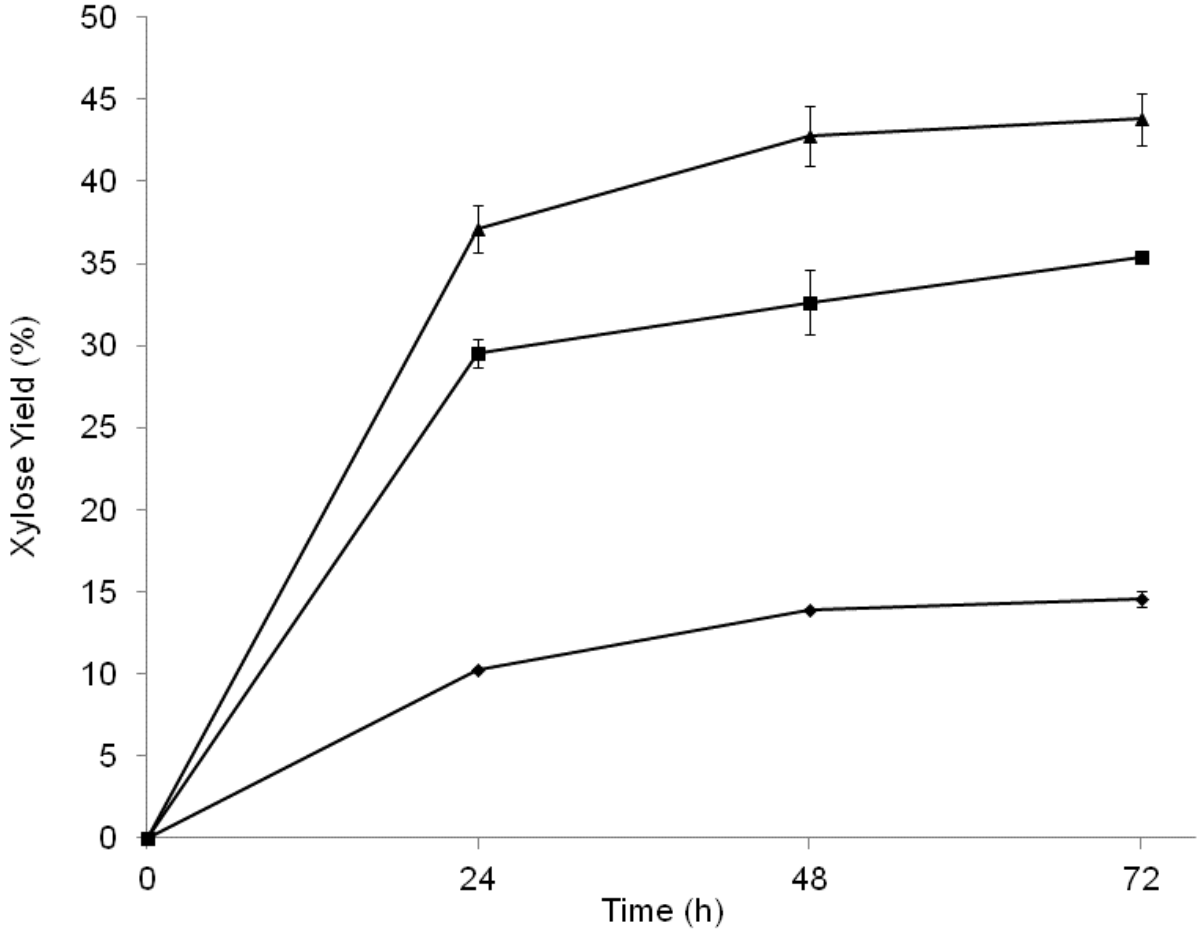

Figure 2: Effect of pelleting on xylose yields from enzymatic hydrolysis of non-pretreated switchgrass.
Data points are average values for triplicate reactors and error bars represent sample standard deviation. ♦: Original; ■: Powder; ▲: Pellet

4.5.3. Effect of pelleting and DA pretreatment on enzymatic hydrolysis of switchgrass

Glucose concentrations during enzymatic hydrolysis of the three different forms of DA-pretreated switchgrass are shown in Fig. 3. The differences in yields between the three forms were not statistically significant at 72 h ($p<0.05$). However, the glucose yields of pelleted and powdered biomass at 24 h were significantly higher (~10%) than from the original biomass, indicating that grinding and pelleting followed by DA pretreatment could increase the initial hydrolysis rate and reactor residence time.

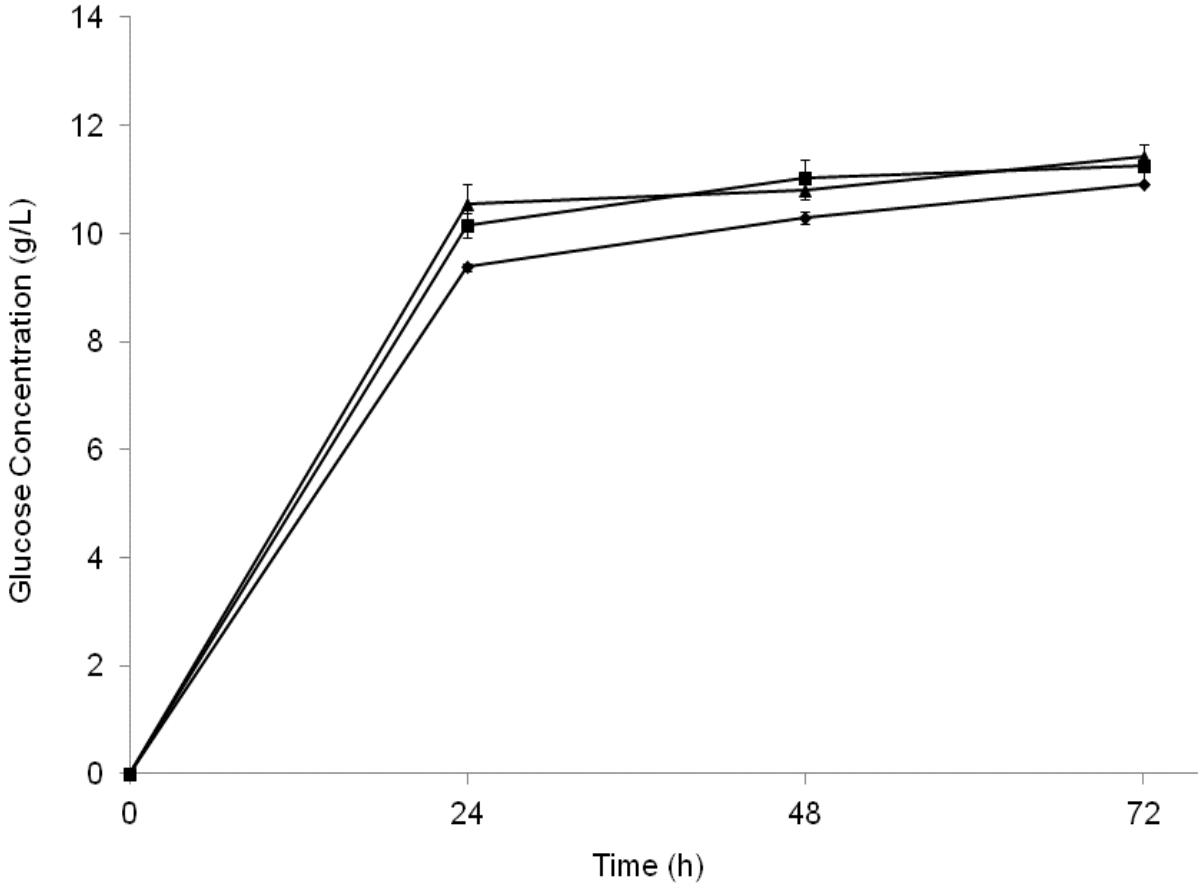

Figure 3: Effect of pelleting on glucose yields from enzymatic hydrolysis of DA-pretreated switchgrass.
Data points are average values for triplicate reactors and error bars represent sample standard deviation. ♦: Original; ■: Powder; ▲: Pellet

Pelleting of switchgrass had no negative effect on the efficacy of DA pretreatment and glucose yields of the three forms were >98% at 72 h. Glucose yields in acidic pretreatments are normally higher than in alkaline pretreatments because of higher removal of hemicellulose and lignin re-localization (Wyman et al., 2011)

4.5.4. Effect of pelleting and SAA pretreatment on enzymatic hydrolysis of switchgrass

Glucose concentrations during enzymatic hydrolysis of the three forms of SAA-pretreated switchgrass are shown in Fig. 4.

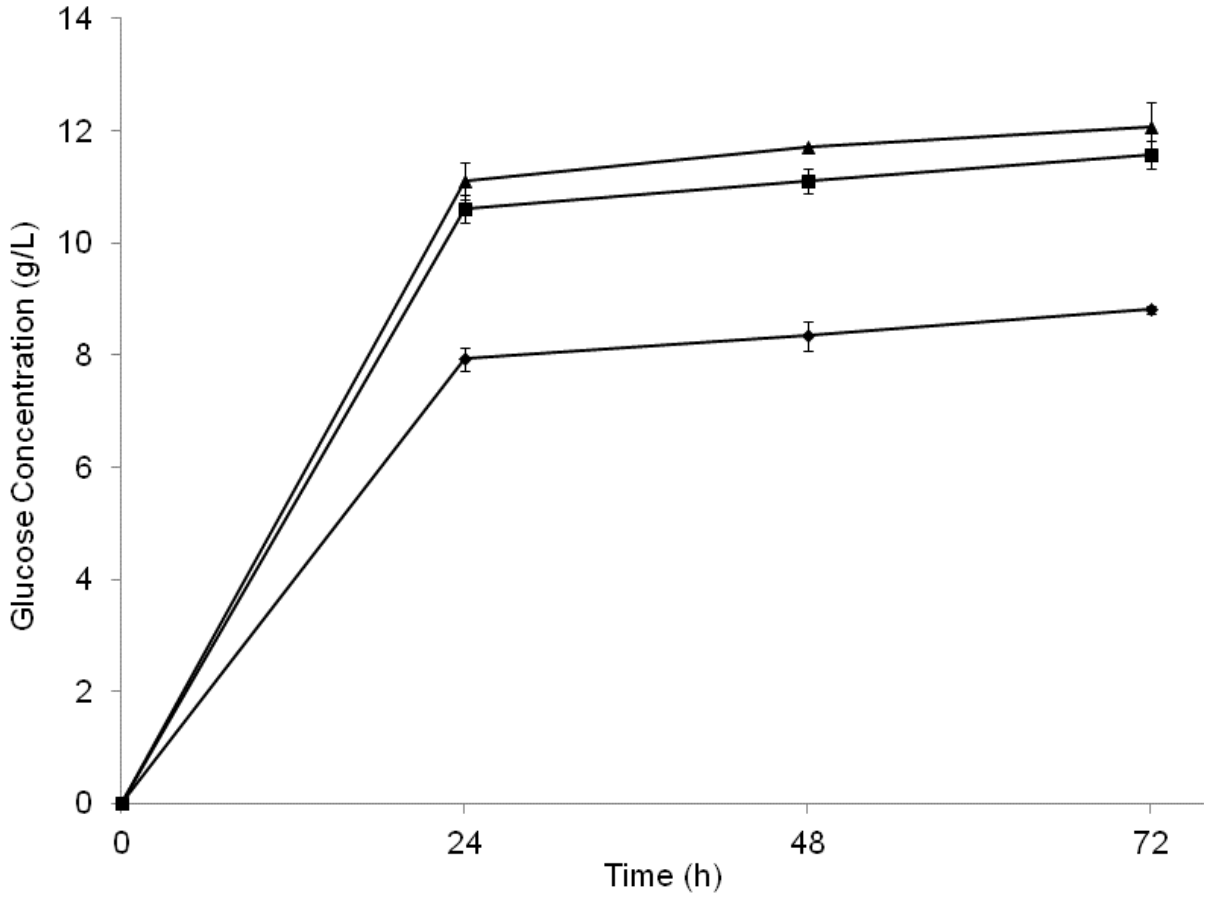

Figure 4: Effect of pelleting on glucose yield from enzymatic saccharification of SAA-pretreated switchgrass.
Data points are average values for triplicate reactors and error bars represent sample standard deviation. ♦: Original; ■: Powder; ▲: Pellet

The rates of glucose production for the different forms followed the same trend and only minor increases in glucose concentrations were seen after 24 h. Glucose yields from pelleted biomass were significantly higher (36% greater) than from original biomass. Differences in glucose yields of pelleted and powdered biomass were not significant ($p<0.05$), indicating that grinding contributed most significantly to yield increases but was not counteracted by subsequent pelleting. Pelleting improved the efficacy of SAA pretreatment and thus may increase its economic feasibility as a pretreatment method.

During the pelleting process, biomass is ground into fine particles generating significant heat prior to compression and pellet formation. In this study, original biomass (D_{90} = 21.7 mm) was ground to a fine powder (D_{90} = 0.5 mm) prior to pelleting and the applied pressure between roller and die was >689 kPa (100 psi). Several studies showed that reduction in particle size increases sugar yields (Dasari and Berson, 2007; Inoue et al., 2008; Koullas et al., 1992; Lamsal et al., 2010). The effects of temperature and shear developed during grinding and extrusion of switchgrass has been shown to have a significant positive impact on sugar yields (Karunanithy and Muthukumarappan, 2010; Karunanithy et al., 2008; Millett et al., 1979). The combined effects of reduction in particle size, shear developed during grinding and compression, and thermal softening or plasticization of lignin that occurred during the process of pelleting likely contributed to improved sugar yields from pelleted switchgrass. Theerarattananoon et al. (2011) showed similar results for glucose yields with biomass pelleting.

The glucose yield of SAA-pretreated original biomass at 72 h was 79.4%. Karki et al. (2011) reported 70.7% glucose yields for switchgrass (same biomass used in this study) with the same pretreatment conditions and cellulase and β-glucosidase loading (without xylanase) after 96 h. Addition of xylanase increased the glucose yields of original biomass. During SAA pretreatment, xylan hydrolysis can enhance access of enzymes to cellulose while xylooligomers also act as strong inhibitors of cellulase activity (Kumar and Wyman, 2009a). Yields following DA pretreatment of switchgrass were significantly higher than those following SAA pretreatment (98% vs. 79%). High xylan retention in the SAA pretreatment may have reduced enzyme accessibility to cellulose despite xylanase supplementation.

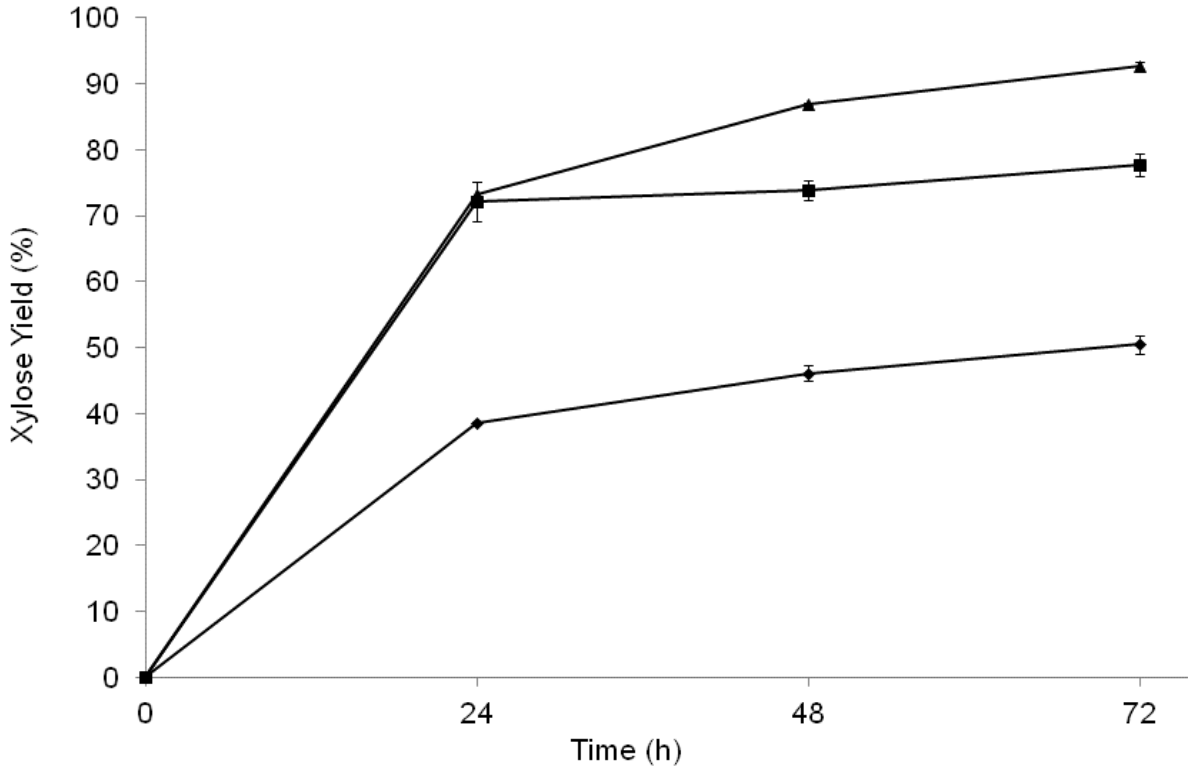

Figure 5: Effect of pelleting on xylose yields from enzymatic hydrolysis of SAA-pretreated switchgrass.
Data points are average values for triplicate reactors and error bars represent sample standard deviation. ♦: Original; ■: Powder; ▲: Pellet

Xylose yields from SAA-pretreated biomass are shown in Fig. 5. The yields from the three forms of biomass followed the same trend as those for glucose production except that differences were more significant between powdered and pelleted biomass. Karki et al. (2011) reported a 48% xylose yield at 96 h for switchgrass at the same pretreatment conditions and with the same cellulase and β- glucosidase loadings. In this study, 6% increases in xylose yields were observed with the addition of xylanase.

This study shows that pelleting of lignocellulosic biomass can be used as a preliminary pretreatment step to increase hydrolysis yields for SAA pretreatments. In addition, results suggest that pelleting processes could allow less severe pretreatment conditions or lower

enzyme loadings to obtain comparable sugar yields. Similarly, ball milling reduced the required hydrolysis enzyme loading and required severity of hot compressed water pretreatment (Inoue et al., 2008). Moreover, pelleting biomass improves feedstock handling, transportation, and storage. This study also confirmed that fine grinding, occurring at an intermediate stage of the pelleting process, could also be an effective preliminary physical pretreatment similar to pelleting. Similar powdered material can be efficiently mass-produced by directly employing fine grinders (e.g., disc mill, ball mill, etc.).

4.6. Conclusions

The increase in sugar yields after switchgrass pelleting was attributed predominantly to the grinding and heating of biomass in the pellet mill prior to actual pelleting. Pelleting itself had no adverse impact on DA pretreatment efficacy and improved the efficacy of SAA pretreatment. Moreover, use of pelleted or finely ground biomass may allow processors to use less severe pretreatment conditions or reduce enzyme loadings and still obtain high sugar yields. Therefore, switchgrass grinding and pelleting may enable more cost-effective downstream processing while providing densification-related benefits such as improved handling, transportation, and storage of biomass feedstocks.

4.7. Acknowledgements

The scientific writing consultation provided by Enrico Sassi (NDSU English Department) is gratefully acknowledged. We are also thankful to Paul Nyren, director of the Central Grasslands Research Extension Center, Streeter, North Dakota, for supplying the switchgrass sample. This study was funded through USDA-NIFA Agreement #2010-34622-20794 and USDA-ARS Agreement #58-5445-8-303.

4.8. References

ASABE Standards. 2007a. S269.4. Cubes, Pellets, and crumbles:definitions and methods for determining density, durability, and moisture content. ASABE, St. Joseph, MI.

ASABE Standards. 2007b. S424.1. Method of determining and expressing particle size of chopped forage materials by screening. ASABE, St. Joseph, MI.

Bailey, M.J., Biely, P., Poutanen, K., 1992. Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 23, 257-270.

Cara, C., Ruiz, E., Oliva, J.M., Sáez, F., Castro, E., 2008. Conversion of olive tree biomass into fermentable sugars by dilute acid pretreatment and enzymatic saccharification. Bioresour. Technol. 99, 1869-1876.

Dasari, R.K., Berson, R.E., 2007. The effect of particle size on hydrolysis reaction rates and rheological properties in cellulosic slurries. Appl. Biochem. Biotechnol. 137, 289-299.

Ghose, T. 1984. Measurement of Cellulase Activity. Pure &Appl. Chem. 59, 257-268.

Hess, J.R., Wright, C.T., Kenney, K.L., 2007. Cellulosic biomass feedstocks and logistics for ethanol production. Biofuels Bioprod. Biorefin. 1, 181-190.

Hu, Z., Sykes, R., Davis, M.F., Brummer E.C., Ragauskas, A.J., 2010. Chemical profiles of switchgrass. Bioresour. Technol. 101, 3253-3257.

Igathinathane, C., Melin, S., Sokhansanj, S., Bi, X., Lim, C.J., Pordesimo, L.O., Columbus, E.P., 2009. Machine vision based particle size and size distribution determination of airborne dust particles of wood and bark pellets. Powder Technol. 196, 202-212.

Igathinathane, C., Ulusoy, U., Pordesimo, L.O., 2012. Comparison of particle size distribution of celestite mineral by machine vision ΣVolume approach and mechanical sieving. Powder Technol. 215-216, 137-146.

Inoue, H., Yano, S., Endo, T., Sakaki, T., Sawayama, S., 2008. Combining hot-compressed water and ball milling pretreatments to improve the efficiency of the enzymatic hydrolysis of eucalyptus. Biotechnol. Biofuels 1, 2.

Isci, A., Himmelsbach, J.N., Pometto, A.L., Raman, D.R., Anex, R.P., 2008. Aqueous ammonia soaking of switchgrass followed by simultaneous saccharification and fermentation. Appl. Biochem. Biotechnol.144, 69-77.

Kaliyan, N., Morey, R.V., 2010. Natural binders and solid bridge type binding mechanisms in briquettes and pellets made from corn stover and switchgrass. Bioresour. Technol. 101, 1082-1090.

Karki, B., Nahar, N., Pryor, S.W., 2011. Enzymatic hydrolysis of switchgrass and tall wheatgrass mixtures using dilute sulfuric acid and aqueous ammonia pretreatments. Biological Engineering 3, 163-171.

Karunanithy, C., Muthukumarappan, K., 2010. Effect of extruder parameters and moisture content of switchgrass, prairie cord grass on sugar recovery from enzymatic hydrolysis. Appl. Biochem. Biotechnol. 162, 1785-1803.

Karunanithy, C., Muthukumarappan, K., Julson, J.L., 2008. Influence of high shear bioreactor parameters on carbohydrate release from different biomasses. American Society of Agricultural and Biological Engineers Annual International Meeting 2008. ASABE 084114. ASABE, St. Joseph, Mich.

Koullas, D.P., Christakopoulos, P., Kekos, D., Macris, B.J., Koukios, E.G., 1992. Correlating the effect of pretreatment on the enzymatic hydrolysis of straw. Biotechnol. Bioeng. 39, 113-116.

Kumar, R., Wyman, C.E., 2009. Effect of enzyme supplementation at moderate cellulase loadings on initial glucose and xylose release from corn stover solids pretreated by leading technologies. Biotechnol. Bioeng. 102, 457-467.

Lamsal, B., Yoo, J., Brijwani, K., Alavi, S., 2010. Extrusion as a thermo-mechanical pre-treatment for lignocellulosic ethanol. Biomass Bioenergy 34, 1703-1710.

Larsson, S.H., Thyrel, M., Geladi, P., Lestander, T.A., 2008. High quality biofuel pellet production from pre-compacted low density raw materials. Bioresour. Technol. 99, 7176-7182.

Mani, S., Tabil, L.G., Sokhansanj, S., 2006. Effects of compressive force, particle size and moisture content on mechanical properties of biomass pellets from grasses. Biomass Bioenergy 30, 648-654.

McLaughlin, S.B., Bouton, J., Bransby, D., Conger, B.V., Ocumpaugh, W.R., Parrish, D.J., Taliaferro, C., Vogel, K.P., Wullschleger, S.D., 1999. Developing switchgrass as a bioenergy crop. in: Perspectives on New Crops and New Uses., (Ed.) J.E. In: Janick, ASHS Press, Alexandria, VA.

McMillan J. D., 1994. Pretreatment of Lignocellulosic Biomass. In: Himmel, M.E., Baker, J.O., Overend, R.P. (Eds.), Enzymatic Conversion of Biomass for Fuels Production. American Chemical Society. Washington DC, pp. 292-324.

Millett, M.A., Effland, M. J., Caulfield, D.F., 1979. Influence of fine grinding on the hydrolysis of cellulosic materials-acid versus enzymatic. Adv. Chem. Ser. 181, 71-89.

Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2005. Determination of extractives in biomass, Laboratory Analytical Procedure(LAP), NREL/TP-510-42619, National Renewable Energy Laboratory, Golden, CO. http://www.nrel.gov/biomass/analytical_procedures.html (accessed January, 2012).

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Croker, D., 2008. Determination of structural carbohydrates and lignin in biomass, Laboratory Analytical Procedure(LAP), NREL/TP-510-42618, National Renewable Energy Laboratory, Golden, CO. http://www.nrel.gov/biomass/analytical_procedures.html (accessed January, 2012).

Tao, L., Aden, A., Elander, R.T., Pallapolu, V.R., Lee, Y.Y., Garlock, R.J., Balan, V., Dale, B.E., Kim, Y., Mosier, N.S., Ladisch, M.R., Falls, M., Holtzapple, M.T., Sierra, R., Shi, J., Ebrik, M.A., Redmond, T., Yang, B., Wyman, C.E., Hames, B., Thomas, S., Warner, R.E., 2011. Process and technoeconomic analysis of leading pretreatment technologies for lignocellulosic ethanol production using switchgrass. Bioresour. Technol. 102, 11105-11114.

Theerarattananoon, K., Xu, F., Wilson, J., Staggenborg, S., McKinney, L., Vadlani, P., Pei, Z., Wang, D., 2011. Effects of the pelleting conditions on chemical composition and sugar yield of corn stover, big bluestem, wheat straw, and sorghum stalk pellets. Bioprocess Biosyst. Eng. doi: 10.1007/s00449-011-0642-8.

Tumuluru, J.S., Wright, C.T., Hess, J.R., Kenney, K.L. 2011. A review of biomass densification systems to develop uniform feedstock commodities for bioenergy application. Biofuels Bioprod. Biorefin. 6, 683-707.

Wyman, C.E., Balan, V., Dale, B.E., Elander, R.T., Falls, M., Hames, B., Holtzapple, M.T., Ladisch, M.R., Lee, Y.Y., Mosier, N., Pallapolu, V.R., Shi, J., Thomas, S.R., Warner, R.E. 2011. Comparative data on effects of leading pretreatments and enzyme loadings and formulations on sugar yields from different switchgrass sources. Bioresour. Technol. 102, 11052-11062.

Yan, J., Hu, Z., Pu, Y., Charles Brummer, E., Ragauskas, A.J. 2010. Chemical compositions of four switchgrass populations. Biomass Bioenergy, 34(1), 48-53.

5. PAPER 2. COMBINED AFEX PRETREATMENT AND DENSIFICATION FOR CELLULOSIC ETHANOL PRODUCTION; EFFECT OF INITIAL PARTICLE SIZE, ENZYME CONCENTRATION, AND STORAGE[1]

[1] Paper 2 will be submitted to the Elsevier journal publication, *Bioresource Technology*, following the completion of my thesis

5.1. Abstract

Switchgrass (SG), corn stover (CS), and prairie cordgrass (PCG) pretreated by ammonia fiber explosion (AFEX) were densified using a novel compaction method (ComPAKco) that operates at comparatively low temperatures and pressures with low energy requirements. Simultaneous saccharification and fermentation (SSF) was performed with AFEX-treated biomass and PAKs to determine the effect of densification after AFEX pretreatment. Densification had no adverse effects on ethanol yields from SG or CS, but reduced ethanol yields from densified PCG by 16%. SSF performed with high enzyme loading (15 FPU/g-glucan, 64 CBU/g-glucan) had 15-20% higher ethanol yields than for low enzyme loading (5 FPU/g-glucan, 21.3/CBU g-glucan). Ethanol yields were not affected by room temperature storage of AFEX-treated biomass or biomass PAKs for 6 months. Reduction in initial biomass particle size from 8-mm to 2-mm before AFEX pretreatment increased the ethanol yields by ~5%, however, grinding of PAKs to reduce particle size after AFEX pretreatment had no improve effect on ethanol yields. AFEX pretreatment and ComPAKco densification could be used for SG and CS densification without negatively affecting ethanol yields; densification of AFEX-pretreated PCG is not advisable.

5.2. Keywords

ComPAKco Densification, Biomass storage, AFEX, SSF

5.3. Introduction

Large centralized lignocellulosic biorefineries face significant challenges related to the logistics of handling, transporting, and storing low bulk density biomass. The challenge of an efficient biomass-to-biorefinery supply chain could be overcome by a network of small preprocessing facilities near the biomass production site (Carolan et al., 2007; Eranki et al.,

2011). Such ‘Regional Biomass Processing Centers’ (RBPCs) may be isolated preprocessing and pretreatment centers. RBPCs may pretreat and densify bulk biomass from local farms, produce stable and dense products, enabling efficient transportation of feedstock to a centralized biorefinery facility. RBPCs minimize the distance and costs for transporting low bulk density biomass. Furthermore, densification could enhance the storability of biomass and its flowability may enable the use of existing grain handling infrastructure.

Biomass pretreatment disrupts the lignin and hemicellulose barriers to make cellulose accessible to enzymes that convert the carbohydrate polymers to fermentable sugars. An effective pretreatment should avoid degradation of carbohydrates, limit formation of inhibitory by-products, and be cost effective. Several pretreatment technologies have been studied for efficient bioconversion of lignocelluloses to ethanol (Tao et al., 2011; Wyman et al., 2011).AFEX pretreatment treats biomass with anhydrous ammonia under moderate pressure (100 to 400 psi) and temperature (70 to 200 °C) for a residence time of 15-30 min; pressure is rapidly released to complete the process (Balan et al., 2009; Bals et al., 2011) . During AFEX pretreatment, lignin is depolymerized and some is redistributed on the surface, hemicellulose is hydrolyzed, and cellulose is partially decrystalized from biomass swelling and subsequent explosive decompression.

AFEX pretreatment offers numerous advantages that are suitable for RBPCs (Carolan et al., 2007). Unlike other pretreatments, AFEX is a dry pretreatment process that preserves and redistributes the lignin on the biomass surface; lignin is available to act as a binder in the densification process. Thus, AFEX pretreatment before densification can improve biomass binding properties allowing densification to be performed at lower pressures and temperatures thereby reducing densification costs (Dale et al., 1999; Sokhansanj et al., 2005).

Various densification systems such as pellet mills, cubers, extruders, briquette presses, roller presses, and tablet presses have been considered for biomass densification (Tumuluru et al., 2011). Significant work has been done to study the densification characteristics of lignocellulosic biomass using pellet mills (Kaliyan and Morey, 2010; Larsson et al., 2008; Mani et al., 2006b). However, pelleting is a relatively expensive and energy intensive process(Mani et al., 2006a; Sokhansanj and Turhollow, 2004). Lower cost biomass densification processes are desirable to lower net costs of feedstock delivery.

A novel densification process was developed by Federal Machine, Inc. (Fargo, ND) that uses a gear and mesh system to compress biomass through a tapered channel between adjacent gear teeth; the process is referred to as ComPAKco densification. This approach involving AFEX pretreatment followed by low pressure biomass compaction could enable effective RBPCs. The capital cost investment for ComPAKco densification has been estimated at less than half of a standard pellet mill. In addition, preliminary trials on ComPAKco densification showed that the system operates at comparatively low pressures and temperature with a lower energy requirement than pelleting. Therefore, RBPCs using AFEX pretreatment and ComPAKco densification could have a positive impact on the low cost supply of feedstocks for large-scale lignocellulosic biorefineries.

Most densification processes generate heat that leads to the melting and subsequent solidifying of lignin allowing it to act as a binder (Kaliyan and Morey, 2010). Alteration of the lignin structure during the densification process could reduce the enzyme's accessibility to the cellulosic fibers. Such impacts could negatively affect the densification-related benefits of feedstock transportation, handling, and storage. Thus, it is necessary to evaluate the interaction effect of densification and pretreatment on hydrolysis yields before evaluating its

impact on feedstock logistics. Densified biomass will likely be stored for several months to maintain a consistent supply of biomass feedstocks throughout the year. However, the literature on the impact of storage of pretreated biomass is limited. Several studies showed the increase in sugar yields with reduction in biomass PS (Dasari and Berson, 2007; Lamsal et al., 2010; Millett et al., 1979). However, the literature on interaction effect of PS reduction and AFEX pretreatment is limited. Therefore, impact of PS reduction on ethanol yields of AFEX-treated biomass was performed to document if reduction in PS is desirable.

When hydrolysis and fermentation are performed in a single step, it is known as simultaneous saccharification and fermentation (SSF). In contrast to separate hydrolysis and fermentation (SHF), where the cellobiose and glucose released during hydrolysis inhibit cellulase activity, monomeric sugars are immediately consumed by microorganisms during SSF maintaining low glucose concentrations and limiting cellulase inhibition (Karimi et al., 2006; Nigam and Singh, 1995; Sun and Cheng, 2002). Additionally, the potential of lower enzyme loadings, reduced capital costs due to single-reactor processing, and reduced risk of contamination due to presence of ethanol makes SSF a better fermentation choice.

In this study, three biomass feedstocks (SG, CS, and PCG) were pretreated with AFEX and densified using the ComPAKco densification method. The objectives of this study were to determine the impact of initial PS, cellulase loading, densification, and storage on SSF yields of AFEX-treated biomass.

5.4. Materials and Methods

Materials and process diagram of this study is shown in Fig.6.

5.4.1. Materials

CS (*Zea mays* L.), SG (*Panicum virgatum* L.) [cv. Sunburst], and PCG (*Spartina pectinata*) [cv. Red River] (MC: 6-7%) were obtained from South Dakota State University (SDSU). Biomass was grown and harvested in South Dakota; samples were ground in a hammer mill (Speedy King, Winona Attrition Mill Co; MN, USA) passing through screen sizes of 2-mm, 4-mm, and 8-mm. Ground biomass was stored in sealed plastic bags at room temperature before shipping to a research group at Michigan State University (MSU) for AFEX pretreatment.

Reagent-grade glucose was obtained from Mallinckrodt Chemicals (Phillipsburg, NJ) and fermentation-grade yeast extract was obtained from Research Organics, Inc. (Cleveland, OH). The enzymes Spezyme CP (cellulase, activity: 59.5 filter paper units (FPU)/mL) was provided by Genencor, Inc. (Rochester, NY) and NS50010 (β-glucosidase, activity: 583.3 cellobiase units (CBU)/mL) was obtained from Novozymes North America, Inc. (Franklinton, NC). The enzyme activities were determined as described by Ghose (1984).

5.4.2. Pretreatment by Ammonia Fiber Expansion

AFEX pretreatment was conducted in the Biomass Conversion Research Laboratory at MSU (East Lansing, Michigan) as described previously by (Balan et al., 2009). Pretreatment of biomass samples (CS, SG, and PCG) were conducted in a 2.0-L pressure vessel (Parr Instruments, Moline, IL, USA) equipped with a thermocouple and pressure sensor. Pre-weighed biomass samples adjusted with the desired moisture content (CS: 60%, SG: 50%, PCG: 40%; dry weight basis) were loaded in the preheated (100 °C) pressure reactor. The required amount of anhydrous ammonia was injected into the reactor at biomass to ammonia loadings of 1:1 for CS, 2:1 for SG, and 2:1 for PCG. The reactor was maintained at a

constant 100 °C temperature and CS, SG, and PCG were pretreated for 15, 30, and 30 min, respectively. Pressure inside the reactor was 200 psi and at the end of the predetermined residence time, the pressure was explosively released. Biomass was unloaded after the pretreatment and allowed to stand overnight in a fume hood to evaporate the residual ammonia. AFEX-treated biomass was stored at 4 °C until use.

5.4.3. Densification

Densification of AFEX-treated biomass was performed using a densification device (Federal Machine Co. d/b/a ComPAKco, LLC; Fargo, ND) to produce the rectangular briquette-like shape called PAKs. Bulk density and true density of PAKs were determined to be ~400 kg/m^3 and ~1400 kg/m^3, respectively, and the moisture content for all PAKs (CS, SG, PCG) were between 11-15%. (Dr. Kasiviswanathan Muthukumarappan, personal communication). Densification is performed using a novel gear and mesh system with comparatively low pressure and temperature with a lower energy requirements.

5.4.4. Inoculum preparation

Saccharomyces cerevisiae (industrial strain obtained from POET, LLC; Sioux Falls, SD) was used in SSF experiments. The seed culture was prepared by inoculating 0.15 g of yeast granules in sterile 50 mM citrate buffer broth containing yeast extract (2 g/L) and glucose (10 g/L). Microbial contamination was prevented by adding 1% (v/v) tetracycline solution (10 mg/mL) prepared in 70% (w/v) ethanol solution. The culture was grown in a water bath rotary shaker (MaxQ 7000, Thermo Scientific; Dubuque, IA) at 150 rpm for 18 h at 37 °C. Fermentation flasks were inoculated with 1% (v/v) inoculum in all SSF experiments.

5.4.5. Fermentation experiments

SSF experiments were performed in 250-mL Erlenmeyer flasks with 100 mL working volume. Biomass was added to a 50 mM citrate buffer of pH 4.8 at an initial glucan loading of 4% (w/v). Enzyme loadings were tested at high and low levels of 15 FPU/g-glucan, 64 CBU/g-glucan and 5/FPU g-glucan, 21.3CBU/g-glucan, respectively. Enzymes were added to the fermentation slurry followed by immediate addition of yeast inoculum. The fermentation flasks were capped with rubber stoppers pierced with syringes (20G x 1 ½" regular level) to maintain anaerobic conditions while releasing excess carbon dioxide. Fermentation flasks were incubated in a water bath rotary shaker (MaxQ 7000, Thermo Scientific) at 37 °C with mild agitation (150 rpm) for 144 h. Samples aliquots (1 mL) were taken aseptically at 0- h (prior to enzyme addition and inoculation), and additional samples were taken at 6 h, 24 h, and every 24 h thereafter. Samples were centrifuged at 13,800xg for 5 min (Galaxy 16 Micro-centrifuge, VWR International; Bristol, CT) and supernatants were filtered through 0.2-µm nylon filter (Pall Corporation; West Chester, PA). Experiments were performed in triplicate. The sugar and ethanol yields were calculated as

$$\text{Sugar yield (\%)} = \left[\frac{\text{glucose or xylose produced (g)} \times \text{hydration factor}}{\text{initial glucan or xylan content in the slurry (g)}}\right] \times 100 \qquad \text{[Eq.1]}$$

$$\text{Theoretical ethanol yield (\%)} = \left[\frac{\text{ethanol produced (g)in the reactor}}{\text{initial glucan content in the slurry (g)} \times 1.11 \times 0.511}\right] \times 100 \qquad \text{[Eq.2]}$$

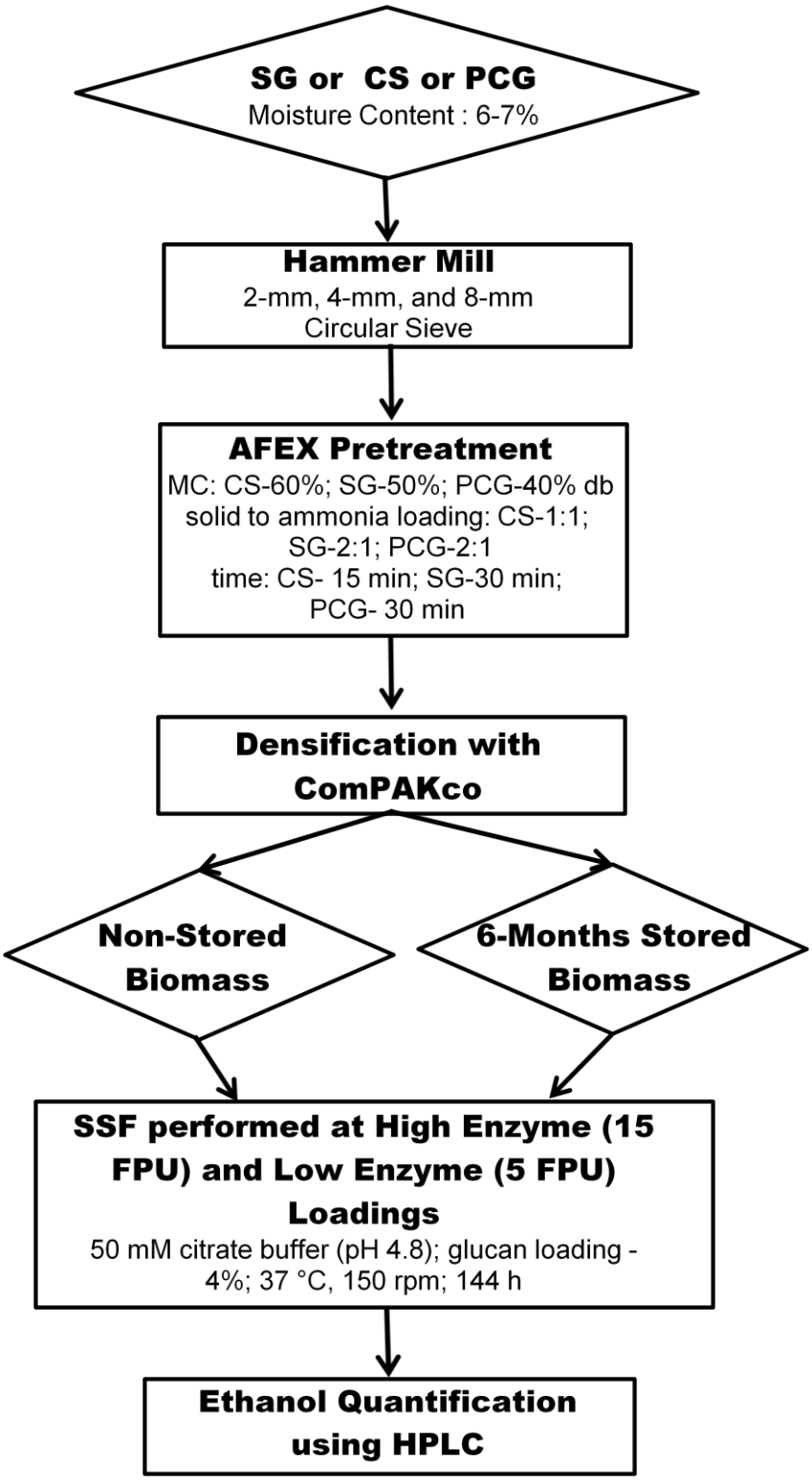

Figure 6: Flow diagram of materials and methods

5.4.6. Compositional analysis

Composition of untreated control and AFEX-treated biomass was performed at the Biomass Conversion Research Laboratory at MSU. The carbohydrates and lignin contents were calculated using the National Renewable Energy Laboratory (NREL) Chemical Analysis and Testing Standard Procedures (Sluiter et al., 2008b). The extractives were removed from the untreated biomass following the NREL Chemical Analysis and Testing Procedures (Sluiter et al., 2008a). Each analysis was performed in duplicates for all the samples.

5.4.7. Measurements of sugars and ethanol

Quantitative analysis for sugars and ethanol in fermentation samples was performed using a HPLC equipped with an autosampler, an isocratic pump, and a refractive index detector (Model 2414, Waters Corporation). The individual sugars were determined using an Aminex HPX-87P (300x7.8 mm) carbohydrate column (Bio-Rad Laboratories; Hercules, CA) and a mobile phase of 18 mΩ NANO pure water at flow rate of 0.6 mL/min at 85 °C. Ethanol was separated using a Bio-Rad Aminex HPX-87H column with a mobile phase of 5 mM sulfuric acid at a constant flow of 0.6 mL/min at 60°C. Sugar peaks (glucose, xylose, arabinose, galactose, and cellobiose) and ethanol were quantified using 4-point external standard curves.

5.4.8. Statistical analysis

The General Linear Model, PROC GLM procedure in SAS (version 9.2, SAS Institute, Inc., Cary, NC, USA) was used to determine least significant difference (LSD) values at $p < 0.05$. The tests of significant difference of overall means were performed for ethanol yields

from biomass in the original, AFEX-treated, and PAK forms for each of the feedstocks (CS, SG, and PCG).

5.5. Results and Discussion

5.5.1. Effect of initial PS on the ethanol yields of CS PAKs

SSF was performed on CS PAKs (2-mm, 4-mm, and 8-mm initial PS) to document the effect of initial PS on ethanol yields (Fig.7.). Results showed that ethanol yields after 144 h were ~5% higher from PAKs from 2-mm PS biomass than from PAKs from 8-mm PS biomass. Reduction in PS increases the enzyme accessibility to the cellulosic fibers and associated hydrolysis and fermentation yields because of the increase in surface area to volume ratio (Mansfield et al., 1999) and reduction in the cellulose crystallinity (Chang and Holtzapple, 2000; Millett et al., 1979) thus increasing fermentation yields.

Ethanol yields were higher for PAKs made from smaller initial PS AFEX-treated biomass, however, preliminary trials on ComPAKco densification study showed better compaction with larger initial PS (J.H. Flaherty, personal communication). A follow-up study was performed to see if grinding PAKs (8-mm initial PS) with a Wiley Mill (Model 4, Thomas Scientific, NJ, USA) could improve ethanol yields. SSF was performed on CS PAKs made from 2-mm and 8-mm AFEX-treated biomass, and on CS PAKs (8-mm initial PS) comminuted to 2-mm to determine the effect of additional grinding after AFEX pretreatment and densification (Fig.8). Final (>72 h) yields of ground 8-mm PAKs were not significantly different than 8-mm PAKs, and were approximately 4% less than yields for original 2-mm PS PAKs. Initial hydrolysis rates, however, were significantly higher for ground PAKs as yields were higher for ground PAKs up to 48 h and yields for all treatments were not statistically different at 72 h. Although, additional grinding to reduce PS after the AFEX

pretreatment and densification would not improve maximum ethanol yields, reactor productivity is higher up to 48 h and ground PAKs performed best in this period. (Chundawat et. al. (2007) also showed that biomass ground before AFEX pretreatment had higher hydrolysis yields than from material ground and washed biomass after AFEX pretreatment, however, the PS of biomass almost 100 folds smaller than PS of biomass in this study.

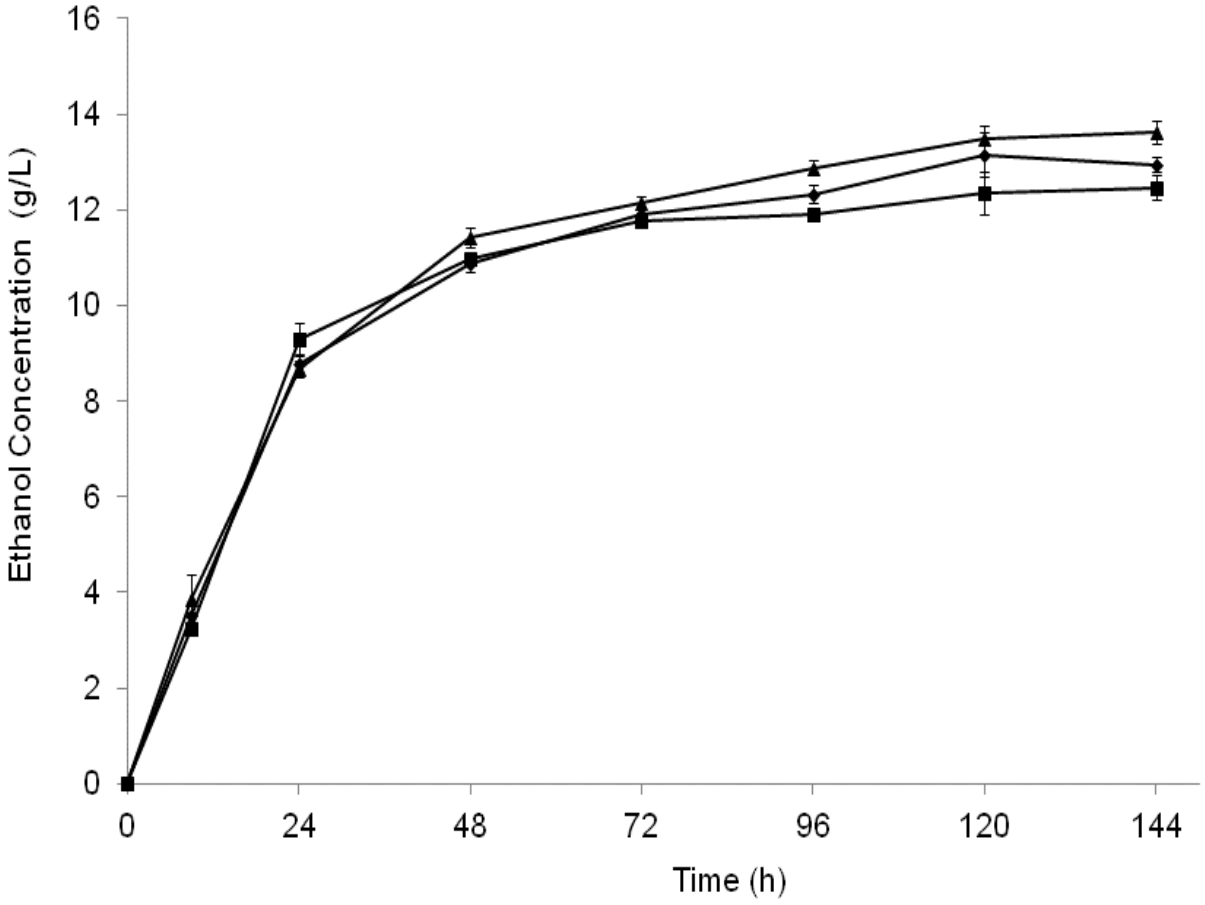

Figure 7: Effect of initial PS before AFEX pretreatment on the ethanol yields of CS PAKs. Data points are average values for triplicate reactors and error bars represent sample standard deviation. ■: 8-mm CS PAKs; ♦: 4-mm CS PAKs; ▲: 2-mm CS PAKs

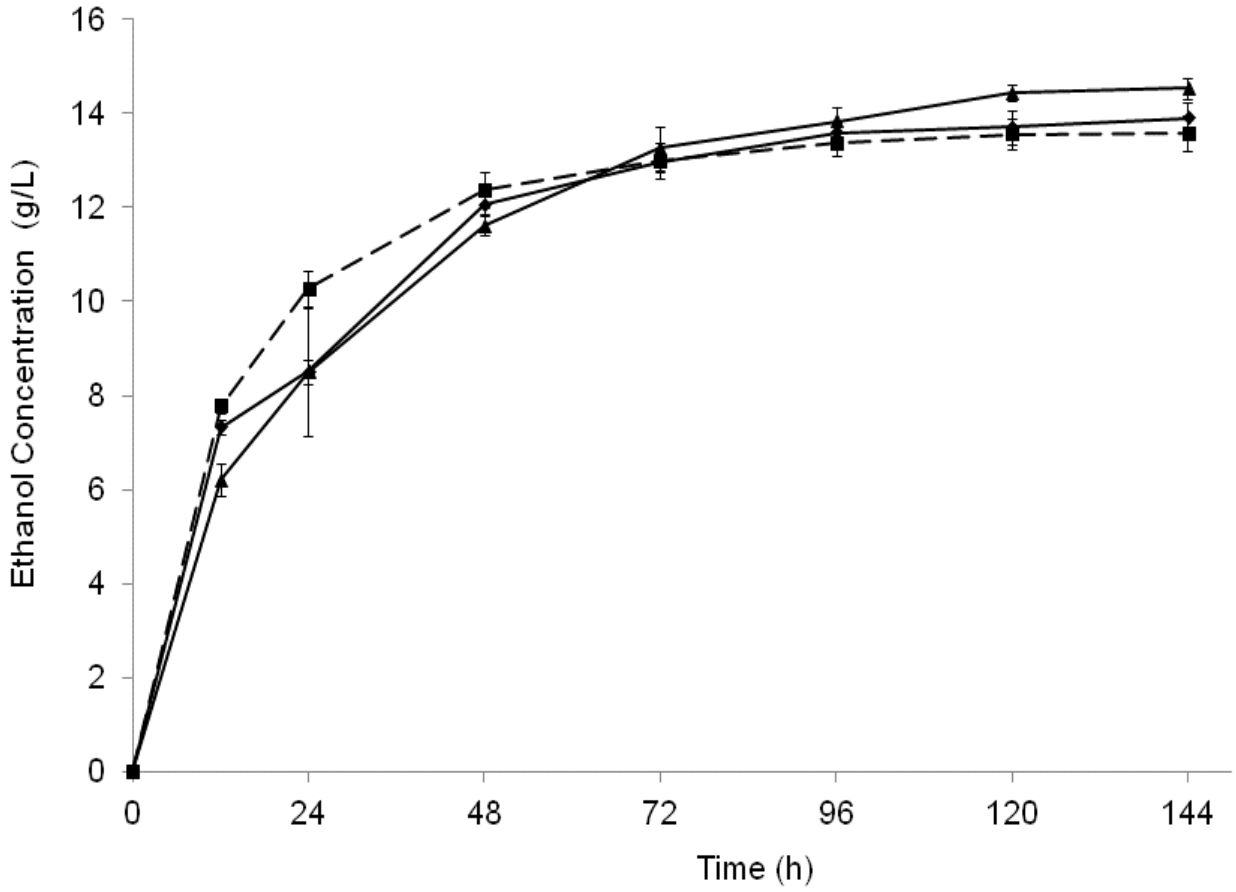

Figure 8: Effect of additional grinding after AFEX pretreatment and densification on the ethanol yields of CS PAKs.
Data points are average values for triplicate reactors and error bars represent sample standard deviation. ■: 8 to 2-mm CS PAKs; ♦: 8-mm CS PAKs; ▲: 2-mm CS PAKs

5.5.2. Effect of densification on the AFEX-treated SG, CS, and PCG

SSF was performed on AFEX-treated SG and SG PAKs to document the effect of post-AFEX densification on ethanol yields. SSF kinetics at high enzyme loadings is shown in Fig.9. Results showed that significant conversion was achieved in the first 72 h for both AFEX-treated SG and SG PAKs. Ethanol concentrations at high enzyme loading, 10.4 g/L for AFEX-treated and 10.3 g/L for PAKs biomass were equivalent to ~45% of theoretical ethanol yields at 144 h; densification of AFEX-treated SG had no adverse effect on ethanol yields.

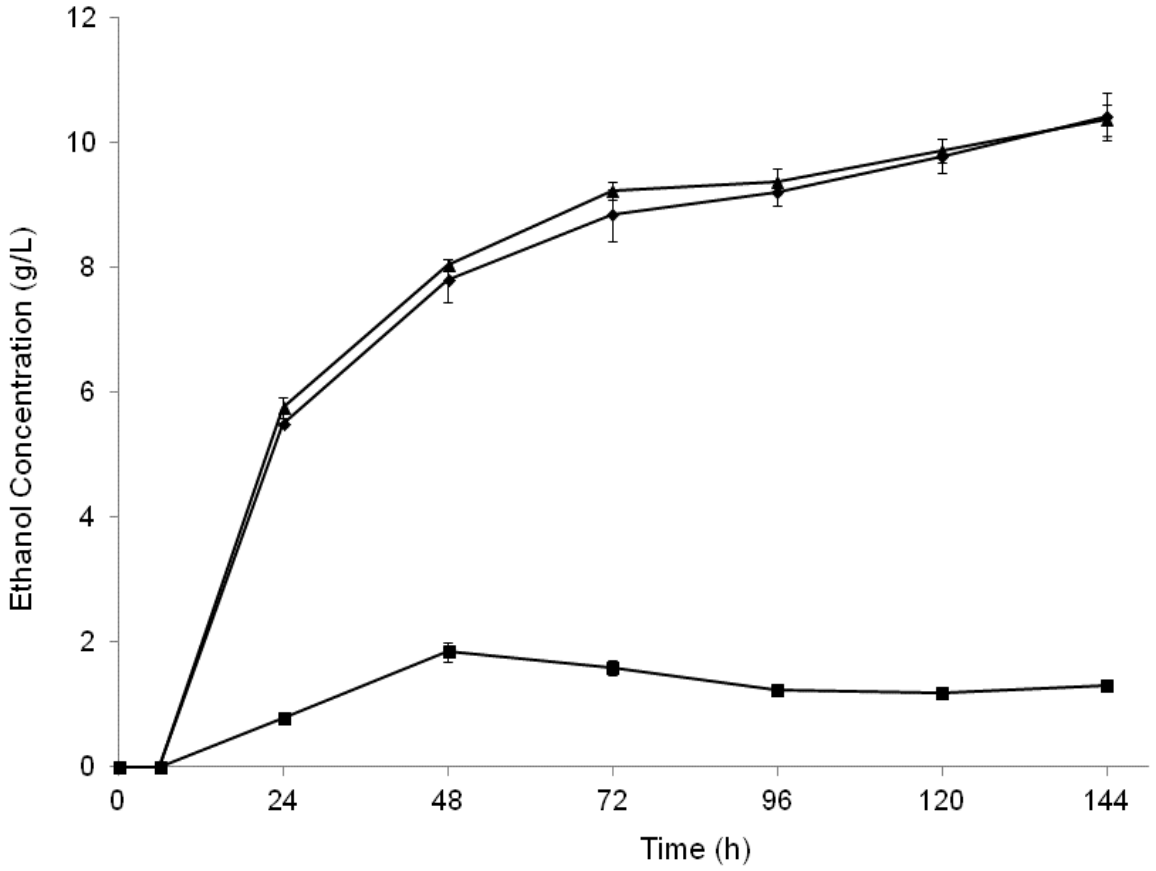

Figure 9: Effect of densification on ethanol yields of AFEX-treated SG.
Data points are average values for triplicate reactors and error bars represent sample standard deviation. ■: Control; ♦: Powder; ▲: PAKs

SSF kinetics at high enzyme loading for CS samples (control, AFEX, and PAKs) are shown in the Fig.10. Ethanol concentrations after 144 h, 14.6 g/L for CS PAKs were slightly higher compared to 13.8 g/L for AFEX-treated CS, however, difference were not statistically significant ($p<0.05$), indicating no adverse effects of densification after AFEX pretreatment.

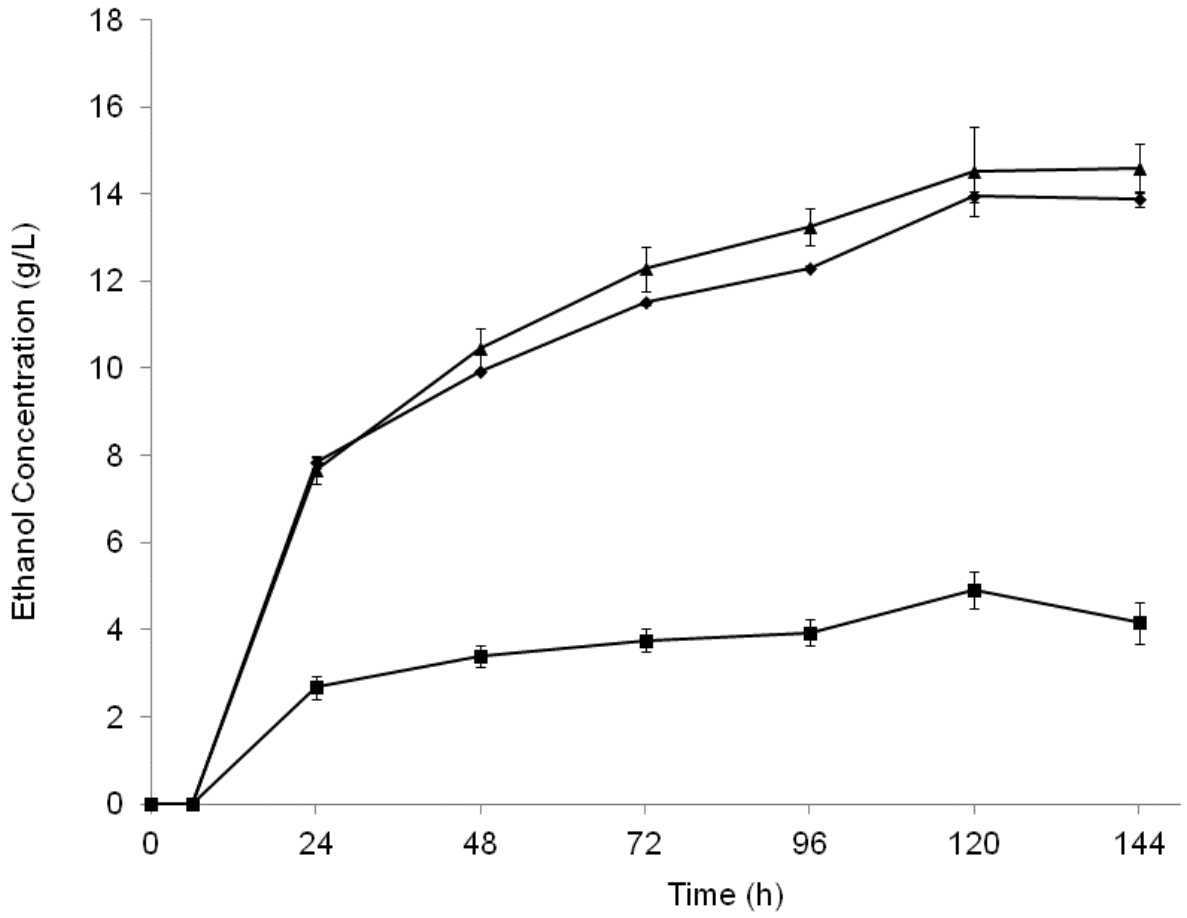

Figure 10: Effect of densification on ethanol yields of AFEX-treated CS.
Data points are average values for triplicate reactors and error bars represent sample standard deviation. ■: Control; ♦: Powder; ▲: PAKs

SSF was performed on PCG samples (control, AFEX, and PAKs) at high enzyme and low enzyme loading. Results showed that comparatively durable densified PCG PAKs could not dissolve in 144 h of fermentation time and ethanol yields for triplicate samples of PAKs had higher variability than seen for CS and SG PAKs (see Fig. 11). Experiments were repeated after grinding the PCG PAKs with a Wiley mill (2-mm screen) at high enzyme loading. SSF kinetics at high enzyme loading before and after grinding PAKs are shown in Fig.11. Ethanol yields of AFEX-treated PCG were ~16% higher than for PAKs, indicating

that densification had a counteracting effect on AFEX-treated PCG that would require additional thermo-chemical pretreatment.

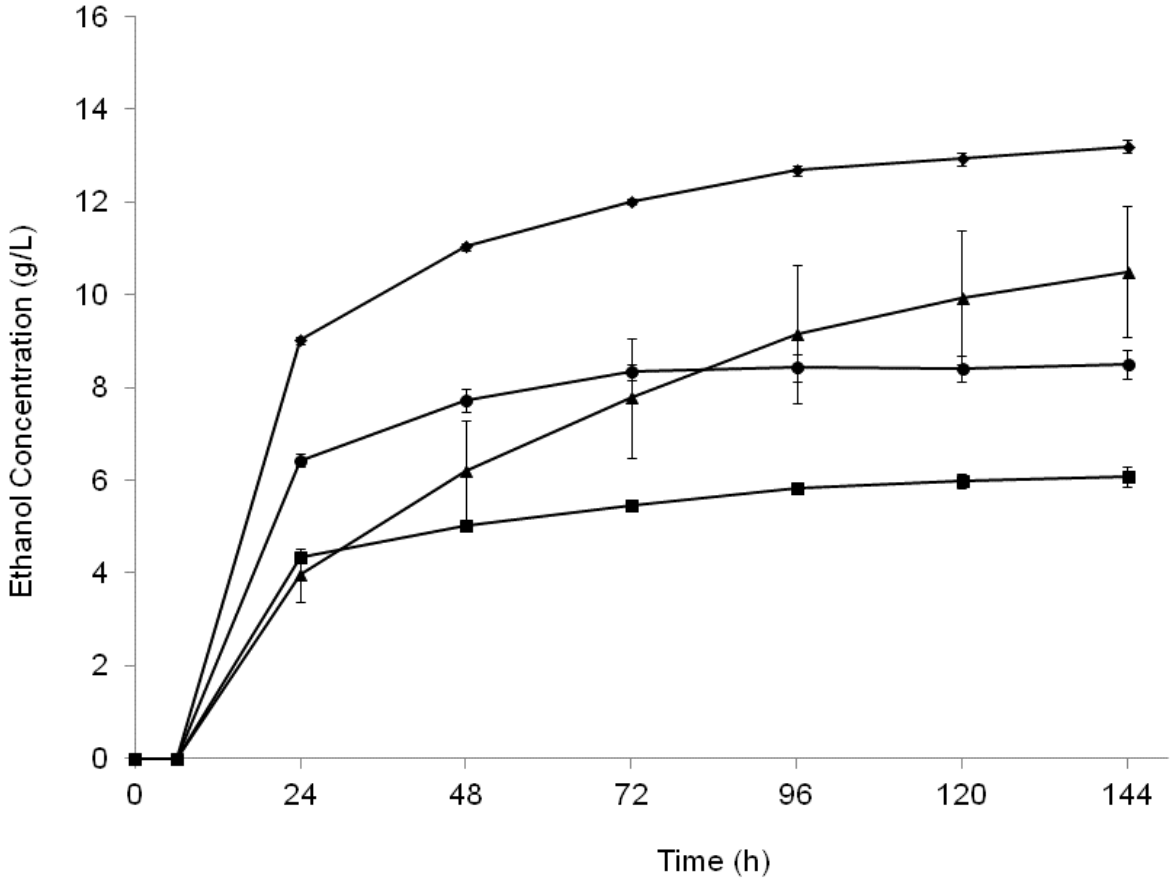

Figure 11: Effect of densification on ethanol yields of AFEX-treated PCG.
Data points are average values for triplicate reactors and error bars represent sample standard deviation. ■: Control; ♦: Powder; ▲: PAKs; ●: Ground PAKs

Densification reduced SSF ethanol yields from AFEX-pretreated PCG. Although the exact reason behind the negative effect of densification in PCG is not known, it could be because of different lignin structure in PCG. Other work has shown that lignin extracts from PCG had significantly lower lignin yields and higher amount of carbohydrates compared to

SG and CS (Cybulska et al., 2012). Higher carbohydrates in the lignin fraction of PCG could be because of a stronger association of lignin with hemicellulose.

The general function group analysis of lignin (by ^{1}H NMR) performed by Cybulska et. al. (2012) showed that syringylpropane and guaiacyl-propane units detected in lignin samples of SG and CS were much stronger than that of PCG lignin. Signals to p-hydroxyphenyl units, C_{α}=O groups, or p-coumaric and ferulic acids were not detected in PCG lignin, however, detected on SG and CS lignin. Also, the signal of ethoxyl groups of PCG was very weak compared to SG and CS lignin.

High temperatures and pressures during the extrusion process also had a negative effect on sugar recovery from PCG (Karunanithy and Muthukumarappan, 2010) while improving yields from SG. PCG soaked in an alkali solution to remove lignin prior to extrusion, however, resulted in increased hydrolysis yields (Karunanithy and Muthukumarappan, 2011). This suggests that PCG lignin could be altered during the mechanical and thermal pretreatment and thus led to reduced enzyme accessibility to cellulosic fibers in PAKs.

The effect of densification was not significant for SG and CS, probably because temperature and pressure developed during ComPAKco densification was not sufficient enough to make significant changes to the biomass structure. Pelleting, a high temperature and high pressure densification process, is shown to have significant positive impact on the hydrolysis yield of SG (Rijal et al., 2012) and CS (Theerarattananoon et al., 2011)

5.5.3. Effect of enzyme loadings on ethanol yields of AFEX-treated SG, CS, and PCG

Final ethanol concentrations (at 144 h) for non-stored AFEX-treated biomass and biomass PAKs at high enzyme and low enzyme loadings are shown in table-4. Results showed that ethanol concentrations were significantly higher ($p<0.05$) for the higher enzyme

loadings except for PCG PAKs. AFEX-treated SG and CS had yields ~20% higher and PCG had ~15% higher yield at high enzyme loadings. Ethanol concentrations from control PCG was not significantly different at high and low enzyme loadings. Control PCG had significantly higher ethanol concentration than control CS and control SG at high enzyme loading.

Others have shown that AFEX pretreatment facilitates lower enzyme loadings. (Teymouri et al., 2005) showed that hydrolysis of AFEX-treated CS had ~95% glucan yield at 15FPU/ g-glucan cellulase loading, and lowering the cellulase loading to 7 FPU/ g-glucan resulted 13% reduction in glucan yield. However, reduction in cellulase loading to 5 FPU/ g-glucan in this study significantly reduced the hydrolysis and fermentation yields and is not recommended for commercial application.

Table 4. Ethanol concentrations of SG, CS, and PCG at 144 h as affected by enzyme loadings and storage

		Non Stored				Stored	
		Low Enzyme Loading		High Enzyme Loading		High Enzyme Loading	
		Ethanol (g/L)	Standard Deviation	Ethanol (g/L)	Standard Deviation	Ethanol (g/L)	Standard Deviation
SG	Control*	0.1	0.05	1.3	0.08	2.0	0.22
	AFEX	5.7	0.25	10.4	0.38	11.2	0.08
	PAKs*	6.2	0.55	10.4	0.25	11.6	0.02
CS	Control	2.8	0.46	4.2	0.48	3.7	0.61
	AFEX*	8.8	0.08	13.9	0.17	15.3	0.17
	PAKs	9.9	0.24	14.6	0.56	15.5	0.04
PCG	Control	5.0	0.19	6.1	0.21	6.0	0.12
	AFEX*	9.9	0.08	13.2	0.14	14.0	0.19
	PAKs#	8.1	0.72	10.5	1.41	10.9	0.10

#Ethanol concentrations of non-stored biomass at high enzyme and low enzyme loadings are not statistically different at $p<0.05$

*Ethanol concentrations of non-stored and stored biomass at high enzyme loadings are statistically different at $p<0.05$

5.5.4. Effect of storage on the ethanol yields of AFEX and PAKs SG, CS, and PCG

Ethanol concentrations for non-stored and stored (6-months at room temperature) biomass in control, AFEX, and PAK forms at high enzyme loadings are shown in table-4. Data showed that storage of AFEX-treated biomass and biomass PAKs for 6 months resulted in modest improvements in yields. Storage of lignocellulosic biomass after cell wall disruption by pretreatment might be more susceptible to chemical degradation or microbial attack, however, this study showed that AFEX-treated and PAKs biomass could be stored for 6 months without any negative effects.

5.6. Conclusions

Ethanol yields from pretreated CS, SG, and PCG can be increased by reducing the initial PS before AFEX pretreatment; however, PS reduction after AFEX pretreatment has no impact on the ethanol yields. ComPAKco densification of AFEX-treated SG and CS could enable RBPCs to overcome the logistic hurdles of biomass handling, transportation, and storage with no impact on the downstream processing steps. Densification of AFEX-treated PCG, however, is not recommended, due to the negative effect on SSF ethanol yields. Low enzyme loading was insufficient for efficient hydrolysis, and AFEX-treated biomass and biomass PAKs can be stored at room temperature for 6 months period without negatively affecting the SSF ethanol yields.

5.7. References

Balan, V., Bals, B., Chundawat, S.P., Marshall, D., Dale, B.E., 2009. Lignocellulosic biomass pretreatment using AFEX, Methods in Molecular Biology. 581, 61-77.

Bals, B., Wedding, C., Balan, V., Sendich, E., Dale, B.E., 2011. Evaluating the impact of ammonia fiber expansion (AFEX) pretreatment conditions on the cost of ethanol production. Bioresour. Technol. 102, 1277-1283.

Carolan, J.E., Joshi, S.V., Dale, B.E., 2007. Technical and financial feasibility of distributed bioprocessing using regional biomass pre-processing centers. J. Ag Food. Ind. Org.(Special issue 5: article 10).

Chang, V., Holtzapple, M., 2000. Fundamental factors affecting biomass enzymatic reactivity. Appl. Biochem. Biotechnol. 84-86, 5-37.

Chundawat, S.P.S., Venkatesh, B., Dale, B.E., 2007. Effect of particle size based separation of milled corn stover on AFEX pretreatment and enzymatic digestibility. Biotechnol Bioeng. 96, 219-231.

Cybulska, I., Brudecki, G., Rosentrater, K., Julson, J.L., Lei, H. 2012. Comparative study of organosolv lignin extracted from prairie cordgrass, switchgrass and corn stover. Bioresource Technology, 118(0), 30-36. doi: http://dx.doi.org/10.1016/j.biortech.2012.05.073

Dale, B.E., Weaver, J., Byers, F., 1999. Extrusion processing for ammonia fiber explosion (AFEX). Appl. Biochem. Biotechnol. 77, 35-45.

Dasari, R., Berson, E.R., 2007. The effect of particle size on hydrolysis reaction rates and rheological properties in cellulosic slurries. Appl. Biochem. Biotechnol. 137-140, 289-299.

Eranki, P.L., Bals, B.D., Dale, B.E., 2011. Advanced Regional Biomass Processing Depots: a key to the logistical challenges of the cellulosic biofuel industry. Biofuels Bioprod Biorefin. 5, 621-630.

Ghose, T. 1984. Measurement of Cellulase Activity. Pure &Appl. Chem. 59, 257-268.

Kaliyan, N., Morey, R.V., 2010. Natural binders and solid bridge type binding mechanisms in briquettes and pellets made from corn stover and switchgrass. Bioresour. Technol. 101, 1082-1090.

Karimi, K., Emtiazi, G., Taherzadeh, M.J., 2006. Ethanol production from dilute-acid pretreated rice straw by simultaneous saccharification and fermentation with Mucor indicus, Rhizopus oryzae, and Saccharomyces cerevisiae. Enzyme Microb. Technol. 40, 138-144.

Karunanithy, C., Muthukumarappan, K., 2010. Effect of extruder parameters and moisture content of switchgrass, prairie cord grass on sugar recovery from enzymatic hydrolysis. Appl. Biochem.Biotechnol. 162, 1785-1803.

Karunanithy, C., Muthukumarappan, K., 2011. Optimization of alkali soaking and extrusion pretreatment of prairie cord grass for maximum sugar recovery by enzymatic hydrolysis. Biochemical Engineering Journal 54, 71-82.

Lamsal, B., Yoo, J., Brijwani, K., Alavi, S., 2010. Extrusion as a thermo-mechanical pre-treatment for lignocellulosic ethanol. Biomass Bioenergy 34, 1703-1710.

Larsson, S.H., Thyrel, M., Geladi, P., Lestander, T.A., 2008. High quality biofuel pellet production from pre-compacted low density raw materials. Bioresour. Technol. 99, 7176-7182.

Mani, S., Sokhansanj, S., Bi, X., Turhollow, A. 2006a. Economics of producing fuel pellets from biomass. Appl. Eng. Agric. 22, 421-426.

Mani, S., Tabil, L.G., Sokhansanj, S. 2006b. Effects of compressive force, particle size and moisture content on mechanical properties of biomass pellets from grasses. Biomass Bioenergy 30, 648-654.

Mansfield, S.D., Mooney, C., Saddler, J.N., 1999. Substrate and enzyme characteristics that limit cellulose hydrolysis. Biotechnol. Prog. 15, 804-816.

Millett, M.A., J., Effland, M.J., Caufietd, D.F., 1979. Influence of fine grinding on the hydrolysis of cellulosic materials-acid vs. enzymatic. Adv. Chem. Ser. 181, 71-89.

Nigam, P., Singh, D., 1995. Enzyme and microbial systems involved in starch processing. Enzyme Microb. Technol. 17, 770-778.

Rijal, B., Igathinathane, C., Karki, B., Yu, M., Pryor, S.W., 2012. Combined effect of pelleting and pretreatment on enzymatic hydrolysis of switchgrass. Bioresour. Technol. 116, 36-41.

Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008a. Determination of extractives in biomass, Laboratory Analytical Procedure(LAP), NREL/TP-510-42619, National Renewable Energy Laboratory, Golden, CO. http://www.nrel.gov/biomass/analytical_procedures.html (accessed June, 2012).

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Croker, D., 2008. Determination of structural carbohydrates and lignin in biomass, Laboratory Analytical Procedure(LAP), NREL/TP-510-42618, National Renewable Energy Laboratory, Golden, CO. http://www.nrel.gov/biomass/analytical_procedures.html (accessed June, 2012).

Sokhansanj, S., Mani, S., Bi, X., Zaini, P., Tabil, L.G., 2005. Binderless pelletization of biomass. ASAE Paper No. 056061, ASAE Annual International Meeting Tampa, FL, USA, July 17-20, 2005. ASAE, St Joseph, MI, USA (2005).

Sokhansanj, S., Turhollow, A.F., 2004. Biomass densification - cubing operations and costs for cornstover. Appl. Eng. Agric. 20, 495-499.

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour. Technol. 83, 1-11.

Tao, L., Aden, A., Elander, R.T., Pallapolu, V.R., Lee, Y.Y., Garlock, R.J., Balan, V., Dale, B.E., Kim, Y., Mosier, N.S., Ladisch, M.R., Falls, M., Holtzapple, M.T., Sierra, R., Shi, J., Ebrik, M.A., Redmond, T., Yang, B., Wyman, C.E., Hames, B., Thomas, S., Warner, R.E., 2011. Process and technoeconomic analysis of leading pretreatment technologies for lignocellulosic ethanol production using switchgrass. Bioresour. Technol. 102, 11105-11114.

Teymouri, F., Laureano-Perez, L., Alizadeh, H., Dale, B.E., 2005. Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover. Bioresour. Technol., 96, 2014-2018.

Theerarattananoon, K., Xu, F., Wilson, J., Staggenborg, S., McKinney, L., Vadlani, P., Pei, Z., Wang, D., 2011. Effects of the pelleting conditions on chemical composition and sugar yield of corn stover, big bluestem, wheat straw, and sorghum stalk pellets. Bioprocess Biosyst. Eng. 35, 615-623.

Tumuluru, J.S., Wright, C.T., Hess, J.R., Kenney, K.L., 2011. A review of biomass densification systems to develop uniform feedstock commodities for bioenergy application. Biofuels Bioprod Biorefin. 5, 683-707.

Wyman, C.E., Balan, V., Dale, B.E., Elander, R.T., Falls, M., Hames, B., Holtzapple, M.T., Ladisch, M.R., Lee, Y.Y., Mosier, N., Pallapolu, V.R., Shi, J., Thomas, S.R., Warner, R.E. 2011. Comparative data on effects of leading pretreatments and enzyme loadings and formulations on sugar yields from different switchgrass sources. Bioresour. Technol. 102, 11052-11062.

6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

Low bulk densities of lignocellulosic biomass and the associated handling, transportation, and storage costs could be the significant challenge facing any cellulosic biorefinery plants in the near future. Densification of biomass could overcome these logistic hurdles and prepares biomass for easy handling, enhances material stability, increases feedstock flowability, and decreases handling, transportation, and storage costs. Biomass densification using pellet mill prior to pretreatment and AFEX pretreatment of biomass followed by ComPAKco densification was studied. Because lignin structure could get altered during interaction of biomass densification and pretreatment such effect could inhibit the accessibility of enzymes to the cellulosic fibers. Pelleting, a high temperature and high pressure densification could potentially degrade the carbohydrates. Such impacts could have negative effect on the densification related benefits of feedstock handling, transportation, and storage.

Pelleting of SG prior to pretreatment or AFEX pretreatment of SG and CS followed by ComPAKco densification could overcome the logistic hurdles of biomass handling, transportation, and storage with no negative impact on the downstream processing steps. However, densification of AFEX-treated PCG is not recommended, due to the negative effect on SSF ethanol yields.

The increase in sugar yields after switchgrass pelleting was attributed predominantly to the grinding and heating of biomass inside the pellet mill prior to actual pelleting. Pelleting itself had no adverse impact on DA pretreatment efficacy and improved the efficacy of SAA pretreatment. Moreover, use of pelleted or finely ground biomass may allow processors to

use less severe pretreatment conditions or reduce enzyme loadings and still obtain high sugar yields that could enable more cost-effective downstream processing.

Ethanol yields from pretreated CS, SG, and PCG can be increased by reducing the initial PS before AFEX pretreatment; however, PS reduction after AFEX pretreatment and densification has no impact on the ethanol yields. Low enzyme loading (5 FPU/g-glucan, 21.3/CBU g-glucan) was insufficient for efficient hydrolysis, and AFEX-treated biomass and biomass PAKs can be stored at room temperature for 6 months without negatively affecting the SSF ethanol yields.

6.2. Recommendations for future work

Further study should be done on the costs associated with these densification technologies to demonstrate the distinct benefits of densification. The benefits of pelleting reported in this study should be validated with further study on the economic feasibility of pelleting. Chemical pretreatment of biomass is an expensive processing step, and its costs are counteracted by its benefits on significant improvements in hydrolysis yields (hydrolysis yields of pretreated biomass are ~75-80% higher compared to non-pretreated biomass). In this study, hydrolysis yield of non-pretreated pelleted biomass was 70% which showed chemical pretreatment of pelleted biomass is undesirable. Therefore, further study could be done on pelleting as a sole pretreatment technique, and hydrolysis yields could be further increased (from 70%) by increasing the enzyme loadings in hydrolysis steps. Also, pelleted biomass could be combined with non-toxic and low severe pretreatments (like hot-water pretreatment, steam explosion, very low acid concentrations) and lower the enzyme loadings during hydrolysis steps. Such combination of pretreatment and hydrolysis steps could have

significant positive impact on economic downstream processing steps and thus reduce the overall cellulosic ethanol production costs.

Further study on the interaction effect of temperature and lignin structure of prairie cordgrass is recommended to see the possible reasons for reduced ethanol yields with ComPAKco densification. Prairie cordgrass PAKs could be pretreated with low severity pretreatment techniques to improve the fermentation yields; however, study on the economic feasibility is also recommended. Further study should be done on increasing the ethanol concentration in the reactor by increasing the enzyme loadings and co-fermenting both glucose and xylose.